"VICTOR BABEȘ" UNIVERSITY OF MEDICINE
AND PHARMACY TIMIȘOARA
FACULTY OF MEDICINE
DEPARTMENT II – MICROSCOPIC MORPHOLOGY

TĂBAN I. OANA

DOCTORAL THESIS

Scientific Coordinator
PROF. UNIV. DR. DEMA ALIS LILIANA CARMEN

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ABSTRACT

NEUROENDOCRINE TUMORS OF THE DIGESTIVE TRACT: A STUDY OF THE MORPHOLOGICAL AND IMMUNOHISTOCHEMICAL FACTORS THAT IMPACT THE PROGNOSIS AND THERAPY OF PATIENTS

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INTRODUCTION

Neuroendocrine neoplasms (NENs) represent complex heterogeneous epithelial tumoral proliferation and include various cryptical entities, some with limited therapeutic options. The tumors often have an unpredictable evolution and seldom reach complete remission after treatment.

Tumors located in the digestive tract represent the majority of neuroendocrine tumors (55%), followed by bronchopulmonary neuroendocrine tumors (25%) [1]. According to SEER (Surveillance, Epidemiology, and End Results), there has occurred a sudden rise in the incidence of digestive neuroendocrine tumors beginning from the year 1973 to the present day. This increase is probably due to progress made in the field of endoscopic and imagistic diagnostic techniques [2]. This conclusion is based on the significant increase in the incidence of localized tumors. SEER data shows that in the decade 2000-2009, neuroendocrine tumors of the digestive tract have represented 0,52% of the newly diagnosed malignancies, with a median incidence of 2,5 cases/100.000 individuals/year [3].

In the past, NENs were classified after the location of the tumor, although many classification systems were based on similar principles. This approach generated a lot of confusion among pathologists. The meeting of a large group of pathologists from the World Health Organisation (WHO), European Neuroendocrine Tumor Society (ENETS), North American Neuroendocrine Tumor Society (NANETS), American Joint Committee on Cancer (AJCC), and College of American Pathologists (CAP) proposed a new classification system for these lesions. During this conference, the decision was made to avoid when talking about primary neuroendocrine lesions the frequently used term *carcinoid* [4,5]. The new classification and grading system of NENs from 2019 is based on the WHO classification of pancreatic NENs from the year 2017. This classification system establishes a clear distinction between well-differentiated neuroendocrine tumors and neuroendocrine carcinomas (NECs). Also, it generates a new category of tumors, the well-differentiated high-grade G3 neuroendocrine tumors [4]. The recent research on neuroendocrine tumors generated a new focus for future studies, to identify the pathogenetic mechanisms, the clinical and pathological associations, and new prognostic and therapeutic markers.

GENERAL PART

Enteroendocrine or neuroendocrine cells (NE) or APUD cells originate from multipotent stem cells and represent the largest and most complex endocrine organ in the human body. These cells secrete a large variety of hormones, for example, gastrin (G cells), ghrelin (P or X cells), somatostatin (D cells), cholecystokinin (I cells), serotonin (enterochromaffin cells), insulinotropic glucose-dependent peptide (K cells) or the glucagon peptide YY (L cells). The secreted hormones are stored in secretory granules and eliminated by exocytosis through the laterobasal membrane, first in the interstitial space and afterward, into the circulatory system [6,7,8].

The density of the NE cells decreases from the duodenum to the rectum. It is greater in the proximal intestine, decreases significantly in the colon, and rises again in

the rectal mucosa. NE cells are classified according to the amino acids/peptides from the secretory granules in three large categories. EC cells, predominantly in the gastrointestinal tract, produce serotonin or 5-hydroxytryptamine. The D cells (delta cells) can be found in the entire gastrointestinal tract and are identified by their positive IHC reaction to serotonin. The L cells can also be identified all along the digestive tract, from the duodenum to the rectum, although they are rare in the segments proximal of the terminal ileum [6,9].

NENs originate in numerous organs and epithelial tissues and include a great variety of tumor types, that differ from an etiological, clinical, morphological, molecular, and prognostic point of view. The NENs classification and grading system from 2019 includes a new category of tumors, the well-differentiated high grade (G3) neuroendocrine tumors. The grading of the lesions (G1, G2, or G3) is defined by the proliferative activity of the tumor cells. The proliferative activity is calculated by evaluating the mitotic rate of the tumor cells and the Ki-67 proliferative index (Ki-67 PI). The mitotic rate is defined as the number of mitosis/2mm^2 and is determined by counting the number of mitosis on 50 microscopic fields of 0,2mm^2. The Ki-67 index is determined by counting the tumor cells with a nuclear expression for the marker among a population of at least 500 tumor cells, located in the areas with the most positive cells („hotspots") at high magnification. If the two factors of tumoral proliferation activity differ, the recommendation is to select the one that indicates a higher proliferation rate. To avoid confusion with the well-differentiated G3 tumors, the new WHO classification specifies that neuroendocrine carcinomas (NECs) do not have a grading system, because these are high-grade tumors by definition. [4].

The TNM staging systems are different for neuroendocrine tumors and gastrointestinal NECs. NECs are high-grade tumors by definition and are classified according to the staging system of carcinomas of the gastrointestinal tract [4,10].

On microscopy, the well-differentiated neuroendocrine tumors present an organoid pattern and are composed of cells with eosinophilic granular cytoplasm and round or oval uniform nuclei, with chromatin dispersed in a „salt and pepper" pattern and small nucleoli. The lesions are classified as well-differentiated neuroendocrine tumors low grade (G1), intermediate grade (G2), or high grade (G3). NECs (small cell or large cell carcinomas) are characterized by a high proliferative rate, with a mitotic rate > 20/2 mm^2 and/or a Ki-67 PI > 20%. The term MiNEN defines a mixt endocrine-nonneuroendocrine tumor, each tumor component representing ≥ 30% of the tumor cells. In MiNENs of the digestive tract, both tumor parts are often carcinomas. These tumors are named MANEC [4,11]. Goblet cell adenocarcinoma (GCA) or ex-carcinoid adenocarcinoma with goblet cells (goblet cell carcinoid according to an early classification system) are amphicrine tumors, located almost always in the appendix. The tumors are composed of mucinous cells, similar to goblet cells, and a variable number of NE or Paneth-like cells.

According to the current guidelines, the NE differentiation of the tumor cells has to be confirmed by using IHC markers like chromogranin A (CgA) and synaptophysin (Syn). Recent studies support the recommendation of evaluating the somatostatin receptor (SSTR) expression in tumors before starting a therapy [8,11].

The symptoms of patients with neuroendocrine tumors are generated by the tumoral expansion process, by the metastatic lesions, or by the hormonal secretion of the tumor cells. Carcinoid syndrome occurs in approximately 30-40% of patients and is characterized by numerous symptoms, like facial flush, diarrhea, or bronchospasm. Fibrosis and nutritional deficit appear later on. In order to diagnose a neuroendocrine tumor, the most frequently used biochemical markers are 5-hydroxyindoleacetic acid (5-HIAA) and CgA [12].

Although the therapeutic approach for tumors between 1 and 2 cm is unclear, for gastric, duodenal, and colorectal tumors ≤ 2 cm the indication is to perform endoscopic mucosal or submucosal resection [12]. For neuroendocrine tumors of the small intestine, surgery is indicated for complete resection of the primary tumor and the mesenteric adenopathy, as well as for clinical staging after an intraoperative evaluation of the peritoneum, liver, and ovaries [13]. NCCN guidelines recommend appendectomy for appendicular lesions under 2 cm and right hemicolectomy for tumors larger than 2 cm [14,15]. Colectomy with regional lymphadenectomy is the recommended approach for neuroendocrine tumors of the colon [12].

The management of tumor progression includes therapy with somatostatin analogues [16,17], targeted therapies with Everolimus [17], Bevacizumab [12] or Sunitinib [18,19], cytotoxic chemotherapy with alkylating agents or anthracyclines or peptide receptor radionuclide therapy [20,21]. Immunotherapy is the last option for patients with end-stage neuroendocrine tumors [12].

SPECIAL PART

1. OBJECTIVES OF THE STUDY AND METHODS

Taking into consideration the numerous changes in the classification system of gastrointestinal NENs, we selected all cases of gastrointestinal neuroendocrine tumors or tumors with neuroendocrine differentiation from the archive of the Pathology Department of Timisoara County Hospital „Pius Brînzeu" from the period 2008-2018. First, we classified the lesions according to the new 2019 WHO classification system [4]. Our study group included 71 cases of NEN, 52 primary gastric, small bowel, appendicular, and colon tumors, and 19 hepatic metastasis of neuroendocrine neoplasms. The lesions were then analyzed using IHC technique for CgA, Syn, p53 protein and Ki-67, to accurately classify and grade the tumors. In the second part of the study, we decided to analyze the molecular profile of NENs by using IHC techniques, with the purpose to identify new prognostic markers and therapeutic targets. In this doctoral thesis, we analyzed the correlations between the results of the IHC reactions for SSTR 2 and 5, anti-PTEN, anti-CXCR4, anti-AKT, and anti-mTOR, and the most important clinical and morphological factors of gastrointestinal NENs. To this day, there is little scientific research available focused on well-differentiated G3 tumors. These tumors were only recently described in the medical literature, their incidence is probably underestimated and until now, there are no standardized treatment methods. Therefore, the purpose of this study is to present the most innovative diagnosis techniques and treatment options, that unfortunately, are unavailable in our country. For the statistical analysis, we used the Hi square test (χ^2) with Yates correction for continuity, and for non-numerical variables, we used the exact Fisher test.

2. RESULTS

The patients (37 men-52,1% and 34 women-47,9%) are between 19 and 88 years old, with a median age of 59,9 years. In our study, most neuroendocrine tumors were diagnosed in women younger than 50 years. 26,7% of the tumors are hepatic metastasis, followed by 18,35% primary tumors of the left colon and rectum. Although we noticed a significant increase in the incidence of neuroendocrine tumors over the last 3 years, the frequency of hepatic metastasis remained the same over the years.

The proliferative activity differed significantly among the study group and increases proportionally with the aggressiveness of the neoplasm (tumor type-Ki-67 PI: neuroendocrine tumors G1-1,2%; neuroendocrine tumors G2-10,5%; neuroendocrine tumors G3-35,7%; NEC-56,2%).

Well-differentiated G1 neuroendocrine tumors represent 38% of the cases in our study. These neoplasms were often diagnosed in young women and in the small bowel (29,1%). Hepatic metastases were present at the time of the diagnosis in 6 cases (22,2%) with a median Ki-67 PI of 1,01%. The median value of Ki-67 PI was 1,2% and varied insignificantly between the location of the primary tumor. From the group of G1 NENs, gastric neoplasms had the highest proliferative activity (median Ki-67 PI of 1,8%). We identified 18 **well-differentiated G2 neuroendocrine tumors** (25,4% of the cases), mostly in men and women past the age of 50 years. Among the well-differentiated G2 neuroendocrine tumors, 22,2% of them have a gastric origin, 16,7% in the right colon, but the most (33,3%) were first diagnosed as hepatic metastasis.

The IHC expression for CgA was positive in 100% of the well-differentiated G2 neuroendocrine tumors, with a diffuse or focal cytoplasmic staining. The IHC reaction for Syn was positive in 50% of the cases. Left colon and rectal tumors were positive for Syn on IHC in 100% of the cases. Small bowel tumors had the lowest median Ki-67 PI (3,4%) and we noticed a statistically significant difference between the median Ki-67 PI in various tumor sites (P=0,01). **Well-differentiated G3 neuroendocrine tumors** (7 cases-9,8%) are more frequent in both men and women older than 40 years. In 4 cases the tumors were first diagnosed as hepatic metastasis, 2 cases in the right colon, and a single duodenal neoplasm. All the metastatic tumors expressed a positive reaction on IHC for Syn. In some cases, the Ki-67 PI was much higher than the median value (35,7%), but there was no statistically significant difference between the median Ki-67 PI in different tumor sites. IHC expression for p53 was negative or only focally positive in less than 25% of the tumor cells. The histological classification of the tumors is very challenging on standard staining methods. Some features are characteristic for this group of neoplasms: focal disturbance of the organoid pattern, marked nuclear pleomorphism, tumor cell distribution in large groups or disorganized trabeculae, small areas of tumoral necrosis, or abundant conjunctive stroma. **NECs** were diagnosed in 12 patients (17%), mostly in men older than 50 years. In 33,3% of cases, the tumors were located in the left colon and rectum. All hepatic metastasis were positive for Syn on IHC. Considering the tumor site, the most aggressive tumors were located in the left colon and rectum (median Ki-67 PI=79,8%; P=0,001). 9 cases of large cell adenocarcinoma were also diagnosed in the same location. In the study group, we identified 3 cases of **MiNEN**, 2 of which combine characteristics of low-grade adenocarcinomas with G2

neuroendocrine tumors. Also, we diagnosed 4 cases of low-grade GCAs or ex-carcinoid adenocarcinomas with goblet cells that were positive for CgA and/or Syn on IHC. In this group of lesions, Ki-67 PI was between 1,8% and 35,2%, with a median value of 20,5%.

Most NENs were diagnosed in the late stages of the disease, with 27 cases as pT3 and pT4 (64,3%) and only 15 cases (35,7%) stage pT1 and pT2. Lymph node invasion was observed in 59,55% of cases and perineural invasion in 33,3%. The median Ki-67 PI was slightly higher in NENs classified as pT3 and pT4 (44 cases-72,1%) than in those diagnosed in stages I and II (17 cases-27,9%; P=0,004). We concluded that there is a statistically significant correlation between the perineural invasion and Ki-67 PI (P=0,02), representing a negative prognostic factor for gastrointestinal NENs.

Among the study group, the IHC reaction **for SSTR2** (clone UMB1, Abcam) was positive in 46 cases (64,8%), with a complete or incomplete membrane staining pattern. 57,9% of the tumor metastasis were positive for the marker, unlike 67,3% of the primary tumors. There is a statistically significant correlation between tumor differentiation and SSTR2 expression on IHC (P=0,0004). Therefore, 96,4% of G1 NENs are positive for SSTR2 on IHC and only 22,7% of the G3 NENs had a positive expression for the marker. We would like to underline the fact that 33,3% of NECs had a positive SSTR2 expression on IHC, a significantly higher percentage than G3 NENs. 50% of GCAs had a positive reaction for SSTR2. All cases of the early neoplastic disease were positive for SSTR2, unlike the tumors that were diagnosed in the late stages III and IV (56,4%), but with no statistical significance. Our results point out that there is a correlation between the SSTR2 expression and the pN stage and a statistically significant correlation with perineural invasion (P=0,04). The tumors with vascular emboli expressed more often the marker (100%), unlike those without lymphovascular invasion (56%).

The immunoreactions for the antibody anti-**SSTR5** (clone UMB4, Abcam) presented cytoplasmic staining in the tumor cells. A positive reaction was observed in 20 cases (28,2%), more often in primary tumors (32,7%) than in tumor metastasis (15,8%). We noticed a statistical correlation (P<0,0001) between the expression of SSTR5 and high-grade NENs (well-differentiated G3 NENs-14,3%; NECs-16,7%). There were no correlations between the SSTR5 expression of the tumors and lymphovascular invasion, perineural invasion, and pN stage.

We identified a positive cytoplasmic, membrane, or cytoplasmic and membrane staining pattern for **CXCR4** (clone UMB2, Abcam) by using a positive external control. The immunoreactivity for the marker of lymphocytes and endothelial cells was considered a positive internal control. The correlation between the CXCR4 expression and the differentiation grade of the tumors is statistically significant; G1 neoplasms presented a weak reaction in 75,5% of the cases, while 63,6% of G3 NENs had a strong positive reaction on IHC (P=0,0002). 7 NENs G1 and 8 NENs G2 had moderate and high scores for CXCR4 expression. There is a significant association between a low score on IHC and the early stages of the disease (P=0,0002), the absence of vascular emboli (P=0,0002), and perineural invasion (P=0,007).

The IHC expression for the anti-**PTEN** antibody (clone 6H2.1, Dako) was evaluated in all of the gastrointestinal NENs included in our research. By using the endothelial cells and nerve fibers as a positive internal control, we noticed cytoplasmic staining in the tumor cells. Loss of PTEN expression occurred more frequently in hepatic metastasis (73,7%) than in primary tumors (50%, P=0,003). Tumors with a weak or negative reaction for PTEN on IHC were located often in the right colon (P=0,03). We noticed a statistical correlation between the weak/absent PTEN expression on IHC and the high grade of the tumors (P=0,003). The expression for PTEN was absent in 4 cases of G1 neoplasms. Appendicular NENs were associated with a high PTEN expression (P=0,02). The immunohistochemical profile was very heterogeneous among the GCA group. A weak/absent IHC expression is correlated to the late stages of the disease (P=0,0007), lymph node metastasis (P=0,008), and lymphovascular invasion (P=0,01).

Immunoreactions for **mTOR** (clone Y391, Abcam) were analyzed by using external control. The staining pattern was cytoplasmic and slightly granular. We noticed a higher proportion of primary tumors that presented a positive expression for mTOR (40,8%) than the hepatic metastasis (16,9%). A strong reaction for mTOR on IHC is statistically correlated with high tumor grade (P=0,0003), advanced clinical stage (P=0,0001), and the presence of hepatic metastasis (P=0,001).

All cases were analyzed by using the monoclonal antibody **pAkt** (clone LP18, Novocastra). By using a positive external control, we were able to observe a cytoplasmic and nuclear staining pattern in the tumor cells. Both primary NENs and hepatic metastasis had a positive reaction for the marker in almost equal proportions (67,3% and 68,4%). Our research concluded that there is a statistically significant correlation between the overexpression of pAkt and high tumor grade (P=0,0003), the early stages of the disease (P=0,002), and pN stage (P=0,04)

3. DISCUSSIONS

NENs are a complex, heterogeneous group of epithelial proliferations, that include various lesions that range from well-differentiated NEN with a slow, subclinical evolution to very aggressive NECs. The latest studies focus on the molecular mechanisms involved in the evolution of the tumors, the clinical and pathological correlations for each tumor site, and identifying new prognostic and therapeutic markers (SSTR, alterations in the signaling pathway PI3K/AKT/mTOR and Notch) [22].

The most recent studies show an increase in the incidence of NENs. In the last 40 years, the incidence of NENs increased 3,6 fold in the United States of America and 3,8-4,8 fold in Europe. The incidence in North America is 2,5-5/100.000 individuals/year, meaning 8000-16000 new cases/year [22] and representing 0,5% of the newly diagnosed malignant tumors and 2% of all malignant lesions of the gastrointestinal tract [1;23]. Neuroendocrine tumors are diagnosed more often in women, rather than men, in a ratio of 2,5:1 [1].

In our research, the gastrointestinal NENs were diagnosed over 11 years in a single medical institution. These tumors were slightly more frequent in men (52,1%) than in women (47,9%).

We noticed a significant increase in the incidence of cases diagnosed after the age of 50 years, mostly in the male population. NENs are more frequent in women when diagnosed in patients under 49 years old. Similar to the data from the literature, we observed a higher frequency of NENs located in the left colon (18,3%) and the right colon (15,5%) [6]. By analyzing the incidence of NENs over each year, we can state that there is an important increase in the number of cases diagnosed after 2016. The incidence of hepatic metastasis was constant over the years.

In 61 cases endoscopy or surgery was performed with a diagnostic or curative purpose. The tumors were classified according to the WHO [4] and AJCC, 8th edition, recommendations [10]. Only 3 tumors (a gastric and two left colon and rectal NENs) were removed by endoscopic polypectomy and were afterward diagnosed as NENs and staged as G1, pT1 tumors. Most NENs were diagnosed in the late stages of the disease. 27 cases were classified as pT3 and pT4 (64,3%) at the time of the diagnosis, in comparison with 15 cases (35,7%) classified as pT1 and pT2. In 54,8% of the lesions, lymph node metastases were already present at the time of the diagnosis, affecting one or more lymph nodes. We observed in 59,5% of cases lymphovascular invasion and 33,35 % perineural invasion.

The effects of somatostatin, a peptidic hormone that inhibits cell growth and hormonal secretion in the tumor cells, are mediated by the interaction of the peptide with the somatostatin receptor family SSTR1-SSTR5 [24,25]. Primary NENs had a positive expression for SSTR2 on IHC in 67,3% of the cases, a much higher percentage than the hepatic metastasis (57,9%). These results are similar to those reported before in the literature [26]. The results of our study were recently published in the scientific journal [27], and demonstrate a strong statistical correlation between the SSTR2 expression and tumor grade (P=0,0004).

The same correlation was noted in well-differentiated neuroendocrine tumors. All the incipient cases of NENs presented a higher percentage than the advanced lesions a positive expression for the IHC marker (56,8%). Our results are supported by the research of Wang et al., who followed 143 patients with gastro-entero-pancreatic NENs and showed that SSTR2 expression is a significant positive prognostic factor [28]. Our data identified no statistical correlation between the SSTR2 expression and pN stage or lymphovascular invasion, although tumors without intravascular emboli presented more frequently a positive SSTR2 expression (100%) than those with no lymphovascular invasion (56%). The absence of perineural invasion is significantly more frequent in SSTR2 positive gastrointestinal NENs (P=0,04). We observed a significant correlation (P<0,0001) between the high SSTR5 expression and tumor type.

Chemokines are a group of cytokines produced by the epithelial and stromal cells [29,30]. They mediate numerous cellular mechanisms involved in cellular signaling and migration. The chemokines mediate their effect by interacting with specific membrane receptors [31]. Well-differentiated G1 tumors had a weak expression for CXCR4 in 75% of the cases (P=0,0002), while G3 NENs presented a high expression on IHC in 63,6% of the cases. Our research confirms the fact that there is a correlation between a low CXCR4 score, the early stages of the disease (P=0,0002), the absence of intravascular tumor emboli (P=0,0002), and the absence of perineural invasion (P=0,007). Our results support the statement that a high CXCR4

expression on IHC is a definite negative prognostic factor for patients with gastrointestinal NENs.

The PTEN protein (phosphatase and tensin homolog protein) is a negative regulatory protein for the signaling pathway phosphatidyl-3-kinase (PI3K)/protein kinase B/Akt [32], that regulates cell growth and survival. IHC analysis revealed that the loss of PTEN expression is more frequent in the case of hepatic metastasis of NENs than in primary tumors (P=0,003). Our results support the research of Wang et al. [33] and Krausch et al. [34]. These studies demonstrated that there is a significant correlation between a weak/absent PTEN expression and high tumor (P=0,003), late stages of the disease (P=0,0007), pN stage (P=0,008)) and lymphovascular invasion (P=0,01). A weak/absent expression of PTEN in tumor cells appears to be a negative prognostic factor. Loss of expression was observed in 4 cases of G1 NENs and one G1 appendicular tumor. These patients need a more aggressive therapeutic approach and a close follow-up.

The signaling pathway PI3K/Akt plays a major role in the process of carcinogenesis. Numerous activatory mutations in the PI3KCA oncogene or inactivating mutations in the PTEN gene occur via this signaling pathway. [35]. The number of cases that expressed a positive reaction to pAkt on IHC was the same in primary NENs as in secondary tumors. This observation suggests that tumor dissemination is not associated with reduced phosphorylation of Akt. Gastric and left colon tumors had the highest number of cases with a moderate/high Akt expression (P=0,0005 and P<0,0001). We noticed a statistically significant correlation between a strong Akt expression and tumor grade (P=0,0001). According to our results, testing for the expression of the inhibitor factors of Akt does not represent a priority in the evaluation of G3 neuroendocrine tumors, but further research is needed with a larger group of patients with G3 NEN.

In our research, we observed a positive expression for mTOR on IHC in a higher proportion of cases than reported before in the literature. This fact is probably due to a large number of high-grade NENs included in our study. The expression of the marker is weaker in the hepatic metastasis than in primary tumors, indicating that the therapy with mTOR inhibitors might be successful only in the case of localized primary NENs. Our results point out that there is a significant correlation between the high expression of the marker and high tumor grade (P=0,0003), tumor location in the right colon (P=0,03), advanced stages of the disease (P=0,0001), and pN stage (0,001). G3 NENs and NECs expressed mTOR in 71,4% and 83,3% of cases, suggesting that the therapy with rapamycin analogues might be indicated in aggressive neoplasms.

CONCLUSIONS AND PERSONAL CONTRIBUTIONS

- In our study, gastrointestinal NENs were more frequent in the male population (52,1%).
- The number of cases diagnosed in the last 3 years has increased dramatically, especially the incidence of the gastric, small bowel, and appendicular NENs. The incidence of hepatic metastasis remained constant over the years, underlining the aggressive character of these tumors.
- The most important clinical, pathological and immunohistochemical factors associated with well-differentiated G1 NENs were: they are diagnosed only in women younger than 50 years, frequently located in the small intestine (29,7%) and left colon (18,5%); the immunoreactions for CgA are often negative in the left colon and rectal tumors (40%); gastric tumors are highly aggressive.
- Well-differentiated G2 neuroendocrine tumors occur in patients of both genders over 50 years old and are located more often in the stomach (22,2%) and the right colon (16,7%).
- Well-differentiated G3 NENs have been often diagnosed after the age of 60, in both genders, especially as hepatic metastasis (57,1%). According to the proliferative activity of the tumor cells, the most aggressive tumors were located in the right colon.
- GCAs were more frequent in older males and they had a median Ki-67 PI of 20,5%, significantly lower than the median Ki-67 PI of G3 neuroendocrine tumors (35,7%).
- The NENs included in our study were diagnosed in the late stages of the disease (64,3% of cases), with lymph node invasion present at the time of the diagnosis in 54,8% of the cases.
- Similar to other studies, we can support the statement that Ki-67 PI is an important prognostic factor for gastrointestinal NENs. Ki-67 PI was strongly correlated to pT stage ($P = 0,01$), clinical stage ($P = 0,004$) and perineural invasion ($P = 0,02$).
- From our research, we can conclude that the pN stage and lymphovascular invasion were not associated with the proliferative activity of gastrointestinal NENs.
- The most aggressive type of NENs is located in the right colon. The lesions are poorly differentiated in 55,6% of the cases and are diagnosed late as pT3 (33,3%) and pT4 (66,7%). These tumors presented lymph node metastasis in 77,8% of cases and tumoral intravascular emboli in 88,9% of the cases.
- Our results support the statement that immunohistochemistry is a valuable, precise, and relatively cheap technique for evaluating the SSTR profile of gastrointestinal NENs.
- Our results support the fact that there is an inverse correlation between the IHC expression of SSTR2, tumor grade ($P=0,0004$), and tumor type ($P<0,0001$). SSTRs can be considered important prognostic factors in the treatment, evolution, and survival of the patients.

- The positive reaction for SSTR2 in MiNENs, NECs, and GCAs suggests that the therapy with SSA in association with surgery and chemotherapy might have optimal results for the survival of patients, although our study needs further validation by other research with a larger patient cohort.
- Our data support the correlation between CXCR4 expression and tumor grade. CXCR4 score on IHC increases proportionally with the aggressiveness of the tumor.
- From our research, we can conclude that a weak CXCR4 expression on IHC was associated with the early stages of the disease (P=0,0002), the absence of intravascular tumor emboli (P=0,0002), and the absence of perineural invasion (P=0,007). The expression of CXCR4 did not correlate with the pN stage.
- The results from this study demonstrated that there is a significant correlation between the loss of expression of the tumor suppressor gene PTEN and the advanced stages of the disease (P=0,0007), lymph node metastasis (P=0,008), and lymphovascular invasion (P=0,01). The loss of PTEN is a negative prognostic factor in gastrointestinal NENs.
- We concluded that there is a significant association between the high expression on IHC for the marker mTOR and older age, tumor sites in the right colon (P=0,03), high tumor grade (P=0,0003), advanced stages of disease (P=0,0001), and pN1 stage (P=0,001). Taking our results into consideration, we propose the hypothesis that mTOR inhibitors are the optimal therapeutic option for patients with aggressive, end-stage, disseminated tumors.
- In our research, we noticed a moderate or intense IHC positive reaction for the marker pAkt especially in the left colon (P<0,0001), gastric tumors (P=0,0005), and high-grade NENs (P=0,0003). The overexpression of the marker is a predictive factor for optimal response to the therapy with Akt inhibitors.
- From our research, we concluded that there is a significant correlation between the high Akt expression on IHC, the presence of lymphovascular (P=0,05), and the pN1 stage (P=0,04). This conclusion supports the important role of Akt activation in the lymphovascular dissemination of NE cells.
- Some of our results have limited value, due to the limited number of cases from some categories of lesions, although there are only a few studies available with a large patient cohort, and second, because our study lacks information about the evolution and survival of the patients. We consider that our results need further validation from other, much larger clinical studies. Our future goal is to expand this study to a higher level.
- The IHC technique adapted for the markers SSTR, PTEN, CXCR4, mTOR, and pAkt is relatively cheap, reproducible, and generates extremely useful information about the negative and positive prognostic factors for the evolution and survival of patients with gastrointestinal NENs. Apart from the morphological criteria, the immunohistochemical tests for specific molecular markers allow an accurate selection and stratification of patients in different risk categories, with major prognostic and therapeutic implications.

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