Looking into digestive MiNENs: subtypes, prognosis and predictive factors

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Abstract

Mixed neuroendocrine and non-neuroendocrine neoplasms (MiNENs) of the digestive system represent a challenging task for both pathologists and clinicians. Their nomenclature has changed several times and their diagnostic criteria, classification, and clinical behaviour have been matter of debate over the past years. Although several attempts have been made to elucidate the pathogenesis and biology of MiNENs, some issues remain still open.

This review will provide:

- a historical background that helps to understand the evolution of the concept and nomenclature of mixed neoplasms
- a revision of the knowledge on this topic, including molecular aspects, to give the reader a comprehensive and practical overview on this challenging field of pathology
- a focus on the diagnostic criteria and on the determination of prognostic and predictive factors
- a description of the different tumour types in the different sites of origin

Key words: mixed neuroendocrine-non-neuroendocrine neoplasm, MiNEN, MANEC, classification, prognosis.

Introduction

The existence of mixed epithelial neoplasms with neuroendocrine and non-neuroendocrine components (MiNENs) is well known to pathologists. Nevertheless, their diagnostic criteria, classification, clinical behaviour, and molecular background have been matter of debate over the past decades and several issues remain still open.

A wide spectrum of possible combinations of neuroendocrine and non-neuroendocrine proliferations have been well documented, spanning from non-neuroendocrine carcinomas with interspersed neuroendocrine cells, through mixed neoplasms with discrete neuroendocrine and non-neuroendocrine components, to classical neuroendocrine neoplasms (NENs) with focal non-neuroendocrine structures.[1] The challenge, for both pathologists and oncologists, is to identify and treat both components, when they are prognostically significant. In this view, MiNENs should be carefully defined in order to be clinically significant. Separate issues are represented by amphicrine neoplasms, which are composed of cells showing both a neuroendocrine and non-neuroendocrine phenotype, and by usual carcinomas without morphologically evident neuroendocrine components showing expression of general neuroendocrine markers.

Such a complex landscape needs to be disentangled using clear cut criteria for diagnosis and classification. In the last years, several attempts have been made to elucidate the clinical and biological meaning of these different combinations, with the main aim to identify solid diagnostic criteria to produce a prognostic classification, useful for patients' management.

In this review, after a historical background that helps to understand the evolution of the concept and nomenclature of mixed neoplasms, we will provide a revision of the knowledge on this topic. In particular, we will focus on the diagnostic criteria, the

determination of prognostic and predictive factors, and we will revise the different tumour types in relation to their site of origin. Last but not least, an update on molecular knowledge will be provided to better understand their pathogenesis.

The history of MiNENs: why do we need a conceptual term?

The first digestive neoplasm composed of an adenocarcinoma and a NEN was described by Cordier in 1924,[2] but it was not until forty-three years later that Lewin proposed the first classification of mixed neoplasms with neuroendocrine and non-neuroendocrine components, suggesting their subdivision into three different subtypes: collision tumours, combined tumours, and amphicrine tumours.[3] Nevertheless, this nomenclature was not universally accepted and several other terms were used over the years to define these neoplasms. The first attempt to standardize the terminology and to provide a prognostic classification of mixed neoplasms of the digestive tract was proposed in 2000 by Capella and co-workers.[4] In the same year, the term "mixed exocrine-endocrine tumour" was introduced by the WHO to define neoplasms composed of a non-neuroendocrine (exocrine) and neuroendocrine component.[5] This heading included the three entities proposed by Lewin (collision, combined, and amphicrine tumours) but, importantly, non-neuroendocrine carcinomas only showing a minority of interspersed neuroendocrine cells were clearly excluded from this category. The main reason at the base of this choice was the knowledge that the presence of scattered neuroendocrine cells in an adenocarcinoma or squamous cell carcinoma is not prognostically relevant. To underline and reinforce this concept an arbitrary cut-off of 30% for each component was established to consider a neoplasm as mixed. This cut-off is still actual, but its biological meaning is controversial and matter of debate. In 2010, the WHO classification of digestive tumours

replaced the term “mixed exocrine-endocrine tumour” with “Mixed AdenoNeuroEndocrine Carcinoma (MANEC)”.[6] This term was probably chosen considering that in most cases mixed “M” neoplasms are composed of adenocarcinoma “A” and neuroendocrine carcinoma “NEC”. Despite this assumption is true, the real spectrum of mixed neoplasms in the digestive tract is wider and includes cases in which other non-neuroendocrine carcinoma types constitute the non-neuroendocrine component (e.g. squamous cell carcinoma in the oesophagus, acinar cell carcinoma in the pancreas, etc...) and cases in which a well differentiated NEN (neuroendocrine tumour- NET), and not a NEC, is present. In these cases, the term MANEC is clearly inadequate to convey the heterogeneity of the possible combinations. More generally, it is worth noting that mixed neoplasms, composed by neuroendocrine and non-neuroendocrine components, the morphology of which depends on the site of origin, can be found in virtually every epithelial organ of the human body. As a whole, these considerations prompted us to find a new term better covering this range of different possibilities, which could also be applied to each body site. After an accurate review of the literature and considering the terminology used to define pure NENs, in 2016 we proposed the term “mixed neuroendocrine-nonneuroendocrine neoplasm (MiNEN)”.[7] The advantage of this terminology resides in the fact that all different entities resulting from the different combinations of various components can be included under this term, which represents an umbrella covering all different entities. Consequently, MiNEN should be regarded as a conceptual category, rather than a specific diagnosis. Indeed, in the pathology report, a diagnosis of MiNEN needs to be better specified including the correct identification and categorization of each component. This new terminology was discussed and accepted during the WHO Editorial and Consensus meeting held in Lyon (April 26th-28th, 2016) for the preparation of the 2017 WHO classification of tumours of endocrine organs.[8] Introduced for

the pancreas, the term MiNEN was retained for all mixed neoplasms of the digestive system and is currently in use.[9]

Diagnosing and subtyping MiNENs: essential tools

Among the different possible combinations of neuroendocrine and non-neuroendocrine epithelial proliferations, the concept of MiNEN includes those neoplasms in which two morphologically recognizable components, namely a NEN and a non-neuroendocrine epithelial neoplasm, coexist in the same tumour mass. In digestive MiNENs, as established in the last WHO classification of digestive system tumours, only malignant non-neuroendocrine components are allowed; in other terms, mixed neoplasms composed by a NEN and an adenoma are not diagnosable as MiNENs. Moreover, each of the two components must represent at least 30% of the neoplastic burden.[9]

The morphological identification of the neuroendocrine and non-neuroendocrine components on haematoxylin and eosin (H&E)-stained sections is the corner stone for the diagnosis. This reflects the concept of MiNEN as a “double faced” or “two for one” problem, in which the pathologist and the oncologist are faced with two interacting neoplastic proliferations, both needing to be managed in diagnostic and therapeutic terms. In this context, one should be aware that MiNEN is not a “hybrid” neoplasm, the biological behaviour of which can be assumed to be the mean of that of the two components. Indeed, from a biological and clinical point of view, the natural history and behaviour of MiNEN is the sum, and not the mean, of the NEN and the non-NEN components, in that they both may progress and metastasize independently, and both deserve to be considered in terms of treatment.[10,11] As for prognosis, it is rather driven by the most aggressive between the two components, and that is why each of them must be characterized, quantified, and graded

separately. The identification of the neuroendocrine component on H&E-stained slides implies the recognition of its well differentiated or poorly differentiated morphology, as we discussed elsewhere.[12] The distinction between well differentiated (NET) and poorly differentiated (NEC) neuroendocrine components is of paramount importance in defining the treatment and the prognosis of each MiNEN. In most of digestive MiNENs, the neuroendocrine component is represented by a large cell or small cell NEC and, accordingly, patients' outcome is ominous.[13] In these cases, the small cell morphology is most frequently present, whereas a large cell component is less commonly observed, paralleling the histopathological aspects of digestive NECs in the various sites.[7] In the few cases of MiNEN including a NET, this should be graded according to the mitotic and/or proliferation index,[9] but the prognosis is usually driven by the coexisting non-neuroendocrine carcinomatous component. It is, thus, evident that the concept of MiNEN allows the prognostic categorization of every single neoplasm, following the accurate characterization of its components. In fact, the introduction of the concept of MiNEN intrinsically allows to delineate a prognostic classification of mixed neoplasms, as the accurate classification of the NEN and the non-NEN allows the oncologist to be aware of the local aggressiveness and of the metastatic potential of each component and to stratify the risk of the patient accordingly. In other terms, MiNENs in which the NEN is represented by a NEC are expected to behave more aggressively than MiNENs including a NET. In the former, the expected outcome is similar to that of pure NEC, whereas in the latter, the prognosis is mainly driven by the non-neuroendocrine carcinomatous component. On this basis, we proposed a theoretical prognostic classification of MiNENs (Table 1), which, however, needs to be validated on large case series.[7]

The non-neuroendocrine part of MiNENs is generally represented by usual carcinomas of the primary site, which must be carefully subtyped and graded in order to define treatment and prognosis. The spatial relationships between the neuroendocrine and non-neuroendocrine components are important to define a MiNEN. The concept of MiNEN implies the assumption that the two components are clonally related[9] and the intermingling of the neuroendocrine and non-neuroendocrine morphology should be observed, at least focally. Nevertheless, we recognize that only the molecular analysis may establish that both components derive from a common precursor, as there are cases of independent neoplasms coexisting in the same site (collision tumours) that can morphologically simulate a MiNEN. As for the quantitative relationships between the two components, digestive MiNEN can only be diagnosed when at least 30% of the tumour mass is represented by one or the other. However, this cut-off is admittedly arbitrary,[9] and it possibly does not have a biological and clinical relevance. This issue is still open and will be addressed at the end of this review.

Immunohistochemistry is mandatory to confirm the neuroendocrine nature of the NEN component and it is also advisable for the accurate subtyping of the non-NEN component. We have discussed elsewhere the application of general and specific neuroendocrine markers to the diagnosis of digestive NENs.[14] In the context of MiNEN, a few points should be recalled and underlined. First, the positivity of the immunostainings for neuroendocrine markers, even for the most specific and sensitive (i.e. chromogranin A and synaptophysin), does not allow the diagnosis of a NEN (and, consequently, of a MiNEN) if a neuroendocrine morphology is not present on H&E-stained slides. This applies, for example, to several cases of usual intestinal adenocarcinomas, which can be diffusely positive for synaptophysin and, to a lesser extent, to chromogranin A, but do not qualify as MiNEN in the absence of a neuroendocrine morphology (Figure 1). A few of these cases, which show a peculiar

morphology with at least focal organoid growth and cell with diffusely amphophilic cytoplasm, represent amphicrine neoplasms, as it can be demonstrated with ultrastructural studies showing the coexistence of neuroendocrine and exocrine granules in the same cells (Figure 2). Amphicrine neoplasms do not belong to the concept of MiNEN and, although they have been well known to the pathologist for a long time, they are still to be characterized in terms of clinico-pathological correlations. This issue will be further discussed, with other unsolved topics, at the end of this review. A second point in the application of immunohistochemistry for general neuroendocrine markers to the diagnosis of digestive MiNEN regards the importance of having at least two positive stains to make a confident diagnosis. Although there are no definitive guidelines on this point, is it highly advisable, unless the morphology is really straightforward, to confirm the neuroendocrine nature of the proliferation with at least two markers,[15] preferably synaptophysin and/or chromogranin A and/or INSM-1. Another major role of immunohistochemistry in diagnosing digestive MiNEN is to define the proliferation grade (G1, G2 or G3) in NET component, according to the Ki67 nuclear stain, expressed as the percentage of positive cells over the total of at least 500 neoplastic cells in the highest labelling areas identified at scanning magnification (Ki67-related proliferation index). In NECs, which are considered high grade by definition, the proliferation rate is much higher than in NETs. Anyway, the Ki67 proliferation index should be recorded in the histopathological report, as there are evidences that it is directly related to patients' survival.[13,16] Finally, the recognition and characterization of the non-neuroendocrine component of MiNENs frequently relies upon the morphological examination of the lesion, as well as on the negativity for general neuroendocrine markers. In a few sites and clinical context, however, the use of immunostainings may give important information. First, as it is detailed in the paragraph on pancreatic MiNENs, an acinar

carcinoma component may be difficult to be distinguished from the neuroendocrine one without the use of an immunohistochemical panel including acinar cell markers (trypsin, BCL10 and others) in addition to general neuroendocrine markers. Second, high grade non-neuroendocrine carcinomas may need an immunohistochemical study in order to be correctly diagnosed and classified, in view of the correct patients' management. For example, in oesophageal MiNENs, the distinction of poorly differentiated adenocarcinoma from a poorly differentiated squamous cell carcinoma may be challenging and heavily impacts on the following treatment. In this situation, the use of histochemistry (i.e mucicarmine, PAS and/or Alcian blue) and immunohistochemistry (e.g. high and low molecular weight cytokeratins, p63, p40, CEA, and others) may help in supporting the correct diagnosis. Third, in case of metastatic presentation of a MiNEN, the analysis of the non-neuroendocrine component with site-specific markers (e.g. transcription factors and/or cytokeratins) may give clues to the primary site, especially when (as it often happens) the neuroendocrine component is represented by a NEC, for which site-specific markers are not useful.[14]

The comparative molecular analysis of the two dissected components has demonstrated that digestive MiNENs derives from a single precursor cell, which undergoes dual differentiation after the first tumorigenic steps. Most of available molecular data have been produced on colo-rectal MiNENs composed of adenocarcinoma and NEC, in which the neuroendocrine and non-neuroendocrine components share common driver genetic aberrations that witness their arising from a single precursor cell. In addition, the two components show exclusive genetic lesions, attesting their progression along different pathways. Interestingly, the early steps of MiNEN tumorigenesis in the colon-rectum involve driver aberration known to be present in the development of colo-rectal adenocarcinoma,

such as *APC*, *KRAS* and *SMAD4* or, in alternative, the genetic pathway involved in microsatellite instability (MSI) (Figure 3).[17-21] In this context, it is worth to say that MSI-driven NECs and MiNENs seem to bear a better prognosis than homologous neoplasms following an APC-driven mechanism.[19,20] Independently from the pathogenetic pathway, the NEC component in these MiNENs shows a higher burden of genetic aberrations than the adenocarcinoma component,[11] also if the genes implied in the genesis of the neuroendocrine phenotype have not been identified, yet. In particular, the loss of function of *TP53* and *RB* seems to represent the most recurrent molecular lesion in the NEC component. A clonal relationship between the two components has recently been demonstrated also in digestive MiNENs composed of adenocarcinoma and NET.[22] Intriguingly, in the rare mixed neoplasms composed by an adenoma and a NET, none of the above-mentioned genetic aberrations have been identified, neither in the neuroendocrine nor in the non-neuroendocrine component, suggesting that other molecular mechanisms are involved in these neoplasms.[23]

MiNEN by MiNEN: the different sites

Oesophagus

MiNENs of the oesophagus and of the gastroesophageal junction represent about 6-16% of all digestive MiNENs and 24% of oesophageal NENs.[11,24,25] They are more frequently diagnosed in males in the sixth decade. In most of cases oesophageal MiNENs are composed of NEC and squamous cell carcinoma. In this site, the distinction between basaloid squamous cell carcinoma and small cell NEC may be difficult, using only H&E-stained sections; thus, immunohistochemical stains for general neuroendocrine markers, high molecular weight cytokeratins, p63, and p40 are mandatory in this context. Infrequently, the non-

neuroendocrine component consists of adenocarcinoma, especially in the distal oesophagus and in the gastroesophageal junction, in association with Barrett's metaplasia.[26] Available molecular data suggest a monoclonal origin of the two neoplastic components from a common precursor stem cell,[27] which show *TP53* mutation, *RB1* deletion or LOH, and amplification of *PIK3CA, PTEN, KRAS, SOX2, DVL3, TP63.* Although exceptional neoplasms combining a NET and an adenocarcinoma have also been reported in Barrett's mucosa,[28] they seem to represent concomitant collision neoplasms rather true MiNENs, since their monoclonal origin has never been confirmed.

Prognosis of oesophageal MiNENs is poor, although it seems better than that of pure oesophageal NECs, with a median survival time of about 20 months.[29] Surgery, especially for localized neoplasms, associated with chemotherapy is the treatment of choice. As for other digestive MiNENs, the Ki67 proliferation index of the NEC component seems to have a prognostic impact.[13]

Stomach

Gastric MiNENs represent about 6-20% of all digestive MiNENs as reported in recent series.[13,24] Male are more frequently affected than females and the age at diagnosis is in the fifth and sixth decade. Macroscopically, gastric MiNENs resemble gastric adenocarcinoma presenting as ulcerated or polypoid lesions measuring from 1.5 to 10.5 cm. They are almost equally distributed in the gastric body and in the antrum.[30] Histologically, they are mostly composed of adenocarcinoma and NEC (for this specific type the term MANEC can be retained), although cases composed of adenocarcinoma and NET have been described as well.[30] It is worth noting that rare cases of adenoma associated with NET, defined as MANETs, have been reported,[23] albeit these neoplasms are not formally considered as MiNENs following the most recent WHO classification of digestive tumours.[9] Gastric MANECs

generally present at advanced stage with lymph node and/or distant metastases. Etiologic factors have not been identified, yet, and the few available molecular data suggest a monoclonal origin of the two neoplastic components of MiNENs composed of adenocarcinoma and either NEC or NET.[22,31-34] MANECs are associated with poor prognosis, which mainly depends on tumour stage and Ki67 proliferative index of the NEC component.[13]

Small intestine

Small intestinal MiNENs are found in the duodenum (mainly located in the in the ampullary region) and, exceptionally, in the jejunum and ileum.

Duodenal MiNEN does not show gender predilection and the average age at diagnosis is about 60 years.[35] Symptoms are generally nonspecific and include pain, weight loss, nausea and vomiting. In some cases, jaundice has been observed, as well.[35] Macroscopically, ampullary MiNEN resembles an adenocarcinoma presenting as fungating or ulcerated lesion with a mean diameter of 2.7 cm. Histologically, most cases are composed of adenocarcinoma and NEC, either of small or large cell subtype. However, rare cases associated with squamous cell carcinoma have been reported, as well.[36] Interestingly, 7 out 14 ampullary NECs reported by Nassar and co-workers were associated with an adenoma, which thus represents a frequent association. However, by definition, these cases are not included in the digestive MiNEN category.[9] One case of adenocarcinoma associated with somatostatin-producing NET was reported, but it morphologically resembled a collision tumour, rather than a true MiNEN.[37] Ampullary MiNEN are aggressive, generally presenting at stage III or IV and are associated with poor prognosis.

Large bowel

The large bowel is the most frequent site of MiNENs along the digestive system, representing 57% of cases.[13]. In addition, colonic MiNENs represent 14-20% of colonic NENs and rectal MiNENs 1-3% of rectal NENs.[10,13,38] There is a male predominance in the sixth or seventh decade of life.[39] Symptoms are generally nonspecific and include weight loss, abdominal pain or bleeding. Since most of cases are metastatic, symptoms may also be related to the metastatic growth.

Macroscopically, colonic MiNENs resemble large bowel adenocarcinomas presenting as polypoid masses or as ulcerated and stenotic lesions, with a mean size of about 5 cm.

Histologically, most of the cases are composed of adenocarcinoma and NEC (Figure 4), although association with squamous cell carcinoma, in both right and left colon, has been observed, as well.[20]

Rare MiNENs composed of adenocarcinoma and NET (Figure 5) have been described in all parts of the large bowel.[7,30] They are large (5 to 7 cm in size) and appear as annular constricting neoplasms in most of cases. Histologically, most tumours are composed of moderately differentiated tubular, papillary, or mucinous adenocarcinoma associated with NET G1 or NET G2. Transitional aspects between the two components, although not prominent, are observed in practically all cases. Interestingly, rare cases associated with either Chron's disease or ulcerative colitis have been reported.[40-42] It worth noting that mixed neoplasms characterized by adenoma and NET can be encountered in the colon and they show an excellent prognosis, thus not requiring large surgical resection.[23]

Three main pathogenetic mechanisms have been described for colonic adenocarcinomas. The first and more frequent mechanism is through the conventional adenoma–carcinoma sequence, which includes a specific sequential *APC*, *KRAS*, *TP53*, *SMAD4*, or *PIK3CA* alterations. The second mechanism includes hypermutant pathway

(microsatellite instability). The third the ultramutant pathway (defective proofreading polymerase with a very high mutation rate affecting very large numbers of genes).[17] Similarly, molecular mechanisms involved in the development and progression of colo-rectal MiNENs composed of adenocarcinoma and NEC include alterations known to be involved in the pathogenesis of large bowel adenocarcinomas such as *APC*, *KRAS*, *TP53*, *BRAF*, *RB1*, and *SMAD4* or, in alternative, the genetic pathway involved in MSI (Figure 3).[17-21] More recently, amplification of *PTGER4* and *MYC* were demonstrated in colonic high grade MiNENs suggesting an intriguing role of these genes in their pathogenesis.[43] Taken together all these data suggest that MiNENs are more closely related to adenocarcinomas rather than to NETs, which show a different molecular background.[18,44,45] Both components of MiNENs show the same genetic alterations, strongly supporting the hypothesis of their clonal origin from a common precursor progenitor cell.[17-19,22,33,34,43]

Colonic MiNEN composed of adenocarcinoma and NEC are aggressive cancers with a median overall survival of 12.2 months. Factors influencing patient's prognosis are the Ki67 proliferative index of the NEC component, the microsatellite instability status and stage.[13,19,20]

Appendix

The appendix is one of the organs in which the change of mixed neoplasm terminology has had the most important impact. In fact, with the introduction of the term MiNEN, the WHO classification of digestive tumours[9] has clearly established that the so-called goblet cell carcinoid, which had been included for a long time in the group of mixed neoplasms (MANECs), is to be considered as a specific subtype of adenocarcinoma with amphicrine features and interspersed neuroendocrine cells.[46] Consequently, the category of appendiceal MiNENs has been reduced to those tumours constituted of two morphologically

recognizable components that are generally represented by adenocarcinoma and NEC. Since for a long time goblet cell carcinoid was considered as MiNEN, most of the literature data regards this entity and clinico-pathologic information on true appendiceal MiNENs is scarce. In two recent epidemiological studies, including cases registered in the SEER database from 1973 to 2012 and from 2010 to 2014,[47,48] the mean age at diagnosis was 58-59.7 years, with equal distribution in both females and males and higher incidence in Caucasians. Most of cases are at stage III or IV at diagnosis with a median overall survival (OS) of 6.5 years, much worse than that of goblet cell carcinoid (13.8 years) and NET (39.4 years).[47] In detail, the reported 1-, 2-, 3-, and 4-year OS was 82.6%, 77%, 73.1, and 62.2%, respectively. Obviously, the OS of patient with stage IV disease is even worse and decreases at 75%, 41.3%, 26.3, and 26.3, respectively.[48]

Pancreas

By definition, pancreatic MiNENs are neoplasms composed of morphologically recognizable neuroendocrine and non-neuroendocrine components, each representing at least 30% of the tumour burden. Consequently, the 20-30% of acinar cell carcinomas showing a minor population of neuroendocrine cells or a non-morphologically recognizable neuroendocrine component should not be considered as MiNENs. Pancreatic MiNENs can arise everywhere in the pancreas and are generally associated with non-specific symptoms due to local tumour growth and/or metastatic dissemination. Pancreatic MiNENs include two main entities: mixed ductal-neuroendocrine carcinoma and mixed acinar-neuroendocrine carcinoma.[49]

Mixed ductal-neuroendocrine carcinoma is rare and accounts for about 0.5-2% of all ductal adenocarcinomas.[50] Males and females are equally affected and the average age at diagnosis is 68 years (range 21-84 years). It is a solid tumour and the reported size ranges between 2

and 10 cm. These neoplasms are composed of ductal adenocarcinoma associated with NEC (Figure 6), although rare cases in which the neuroendocrine component was constituted by a NET have been described.[51-53] However, they morphologically resemble collision tumours rather than true MiNENs and their monoclonal origin has never been demonstrated. The two components of mixed ductal-neuroendocrine carcinomas are frequently intermingled, but sometimes they appear more clearly separated. The immunohistochemical profile of the adenocarcinoma component includes the typical phenotype of ductal adenocarcinoma such as the expression of CEA, MUC1 and/or MUC2. The NEC component expresses synaptophysin and chromogranin A. The most important differential diagnoses are ductal adenocarcinomas with entrapped islets and PanNETs with entrapped ductules. Morphology and immunohistochemistry are of great help to solve these issues. In ductal adenocarcinoma with entrapped islets, islets show the characteristic ovoid shape with regular contours without atypical cells. It is worth noting that in cases associated with chronic pancreatitis, pancreatic islets may appear increased in number and size, mimicking a neoplastic proliferation. In these difficult cases, immunohistochemistry is useful because normal islets cells show the well-known and specific intra-insular expression of the four pancreatic hormones (Figure 7). Conversely, in MiNENs, the neuroendocrine component does not show a regular ovoid structure, but it is rather trabecular, and only one or a predominant cell line is found. In PanNETs with entrapped ductules (Figure 8), morphology showing the lack of duct cells atypia, associated with the regular shape of ductules is of help. Entrapped ductules generally do not show aberrant p53 immunostaining, are positive for SMAD4 and nuclear Ki67 expression is restricted to scattered cells.

Due to their rarity, molecular data are scarce and not definitive. Mixed ductal-neuroendocrine carcinomas are frequently metastatic to lymph nodes and liver at the time

of diagnosis and are associated with a dismal prognosis. Indeed, patients rarely survive more than 3 years: the reported 2- and 5-year survival rates are 25% and 0%, respectively.[49,54]

Mixed acinar-neuroendocrine carcinomas are neoplasms displaying morphologically distinguishable acinar and neuroendocrine components (Figure 9). They are rare accounting for about 15-20% of all pancreatic acinar cell carcinomas.[55,56] They are large (4 to 8 cm) and generally well-delimitated neoplasms with a fleshy and focally necrotic cut surface. Macroscopically, they resemble pure acinar cell carcinomas and the mixed nature of the neoplasm is only detected histologically and confirmed using immunohistochemistry. The neuroendocrine component is positive for general neuroendocrine markers and negative for acinar cell markers, whereas the acinar component has an opposite phenotype. However, it must be recalled that the expression of synaptophysin is not exceptional in ACC,[56] further underlining the importance of a double positivity for neuroendocrine markers to diagnose a NEN. Among acinar cell markers, trypsin and BCL10 are the more specific and sensitive and their simultaneous use detects almost 100% of cases. It is worth noting that BCL10 protein is not actually expressed by acinar cells. The BCL10 immunoreactivity depends on the homology between the amino acid sequence 156 and 205 of the COOH terminal portion of BCL10 protein and the sequence between amino acid 564 and 608 of carboxyl ester lipase (CEL). For this reason, the use of the monoclonal antibody directed against the COOH terminal portion of the BCL10 protein (clone 331.3) is crucial for the diagnosis of ACC.[57] The recognition of an ACC components in an apparently pure PanNEN is important because mixed acinar-neuroendocrine neoplasms are clinically more aggressive than pure PanNETs. Due to some overlapping features between PanNENs and ACCs, the differential diagnosis may be sometimes difficult. In this context, it is strongly suggested to perform BCL10 and trypsin immunohistochemistry in all neuroendocrine-looking pancreatic neoplasms that

show a high mitotic index, abundant necrosis and evident nucleoli. No association with genetic syndromes has been documented to date. These tumours seem to share the genetic changes observed in pure acinar cell carcinomas, such as alterations in the APC/β-catenin pathway and *BRAF* fusions.[58,59] Characteristic mutations that can be found in pancreatic NETs (*DAXX*, *ATRX*, and *MEN1*) have not been observed. c-MYC alterations have been recently suggested to be involved in mechanisms leading to the neuroendocrine differentiation of acinar cell carcinomas.[60] Surgical resection, together with tumour stage, is the most important prognostic factor and the reported 5-year survival is 30-50% for operated patients.

In addition to these two mains pancreatic MiNEN types, extremely rare neoplasms showing ductal, acinar, and neuroendocrine differentiation have also been reported and defined as mixed ductal-acinar-neuroendocrine carcinomas. This is a very rare and not well-documented neoplasm and histologically is very similar to mixed acinar-ductal carcinomas. Since in the reported cases the neuroendocrine component was not identifiable morphologically, but detected with immunostaining for neuroendocrine markers, they should be considered as a variant of ACC, rather a separate entity of pancreatic MiNEN.[61]

Gallbladder and biliary tree

Gallbladder MiNENs are rare, representing about 10% of gallbladder malignancies and about 2% of all hepatobiliary carcinomas.[62] However, recent findings suggest that they are more frequent than previously described, as a non-neuroendocrine component, mostly adenocarcinoma, is recognizable in a third of diagnosed gallbladder NECs.[63] The median age of patients is 65 years (range: 34-85 years) and females seem more frequently affected than males. Symptoms are generally non-specific and, for this reason, they are incidentally

discovered as intracholecystic lesions during imaging investigations for other reasons. At the time of diagnosis, 65% of tumours are limited to the gallbladder wall, with involvement of the serosa surface in 21% of the cases. Infiltration of adjacent organs is rare and observed in about 30% of patients. Liver, peritoneal, nodal or, rarely, distant metastases are observed in about 50% of cases at the time of diagnosis.[64]

Macroscopically, gallbladder MiNEN is indistinguishable from a pure adenocarcinoma. Histologically, most cases are constituted by an adenocarcinoma, with different degree of differentiation, associated with small cell NEC. Rarely, other components such as squamous cell carcinoma or carcinoma with sarcomatoid or osteosarcomatous differentiation have been described. In some cases, an association with an intracholecystic papillary neoplasm, which represents a pre-invasive gallbladder lesion, has been reported suggesting a possible evolution from a pre-malignant component. This seems to be also supported by molecular analyses, although performed in a very limited number of cases. Available molecular findings suggest that the different components of gallbladder MiNENs derive from the same precursor progenitor cell, as observed in other digestive MiNENs. The most important driver seems the point mutation in *TP53*, which has been found in both malignant neuroendocrine a non-neuroendocrine components as well as in the pre-invasive intracholecystic papillary neoplasm.[64]

Disease stage, namely the presence of metastases, seems the most important prognostic factor. Indeed, it has been reported that about 80% of patients with non-metastatic MiNENs limited to the gallbladder are alive after a mean follow-up time of 12 months, compared to 22% with metastatic disease.

MINENs occurring in the biliary tree and in the hilar portion of the hepatic duct are extremely rare. Since they have been frequently observed in association with inflammatory

hepatobiliary diseases, it has been suggested that a pre-existing chronic inflammation, associated or not with lithiasis, may play a pathogenetic role in the development of such neoplasms. Morphologically, as observed in the gallbladder, the non-neuroendocrine component is generally represented by an adenocarcinoma with variable degree of differentiation. The neuroendocrine component is generally deeply located and is constituted by NEC or, less frequently, by a NET G2.[62]

Liver

Very rare cases of hepatic MiNENs have been reported in the literature. The average age at diagnosis is 65 years (range 43-73 years) and males are more frequently affected than females. Abdominal pain is the most frequent symptom and patients generally present with metastatic dissemination to lymph node or distant (lung, bone) sites. The prognosis of is dismal since disease-related death generally occurs after a mean follow-up time of 6 months.[7]

In most of the cases, the neuroendocrine component is represented by a NEC (both small and large cell subtype); in only one reported case a NET was present. The non-neuroendocrine component is most frequently represented by a hepatocellular carcinoma (HCC), with only very rare reported cases of cholangiocarcinoma.[7] In some neoplasms, the HCC component was intermingled with the NEC component, while in one case the two components were completely separated suggesting for this case a collision tumour rather than a true MINEN. The histogenesis of these mixed hepatic neoplasms is unclear but some authors suggested that the neuroendocrine component represents the result of the neuroendocrine differentiation of a pre-existing HCC,[65,66] although this theory needs to be finally demonstrated.

Open issues

In their almost one century-long history, mixed neoplasms with NEN and non-NEN components have raised several challenges to both pathologists and oncologists. The application of immunohistochemistry and molecular genetic analysis, along with the evolving knowledge and understanding of the clinico-pathological correlations, has led to improved classification criteria and have shed light on many important biological and diagnostic aspects. The introduction of the term MiNEN reflects, under many points of view, the modern approach to these neoplasms and allows to take into account their heterogeneity, as well as to harbour future evolutions in terms of both risk stratification and of response to treatment prediction.[7] Nevertheless, and plainly, a number of issues regarding MiNENs remain still open and a few of them deserve to be pointed out here.

Amphicrine carcinomas

The application of immunohistochemistry to histopathological diagnostic practice has surely the merit of having unveiled or, better, confirmed the presence of neuroendocrine differentiation in a number of mixed neoplasms. However, the indiscriminate use of immunostains for general neuroendocrine markers in the diagnostic workup of morphologically non-neuroendocrine neoplasms (i.e. usual digestive adenocarcinomas or squamous cell carcinoma) may lead to a number of misdiagnosis, as we already examined in this review. A different issue is represented by hybrid neoplasms, which show coexistent morphological, immunohistochemical and ultrastructural features of both neuroendocrine and exocrine differentiation in the same cells (Figure 2). Such neoplasms, that can be find in digestive and extra-digestive locations, are known since the early 1980s and are named *amphicrine*.[67] Their relationship with NENs and MiNENs has been a matter of debate for a long time,[3,7] particularly in the appendiceal location, where they are fairly common and have

been misnamed as goblet cell carcinoids for a long time. The new designation *appendiceal goblet cell adenocarcinoma*[46] does not convey their amphicrine features, but at least it avoids the possible misinterpretation of such potentially aggressive neoplasms as low grade NENs. Amphicrine neoplasms have also been described in the stomach and in the bowel. A recent study on the clinico-pathological, immunohistochemical and molecular features of a series of 10 cases (8 gastric and 2 intestinal neoplasms) has shown that amphicrine neoplasm is a unique entity with distinct biological and histological features.[68] In this study, the histological three-tiered grading proposed by Yozu and co-workers for appendiceal goblet cell adenocarcinoma[69] proved to be effective also in gastric and intestinal amphicrine neoplasms for predicting prognosis, also independently from disease stage.[68] The pan-cancer transcriptome analysis revealed that amphicrine neoplasms share similarities with adenocarcinomas, but not with NENs. Unfortunately, the exact nature of NENs included in the study was not stated and it is not clear whether they were NETs or NECs.[68] As a whole, we believe that the available data discourage the inclusion of amphicrine neoplasms in the concept of MiNEN, for both biological and clinical reasons. Practicing pathologist should be well aware of the differences between MiNEN, amphicrine neoplasms and non-endocrine carcinomas with immunohistochemical expression of neuroendocrine markers and of the clinical meaning of each of these entities (Figure 10).

The cut-off to define a MiNEN

The diagnosis of MiNEN has relied for years on the relative proportions of the neuroendocrine and non-neuroendocrine components, each needing to reach a threshold of 30% of the whole neoplastic volume to comply with the definition stated by the WHO classifications.[5,6,9] The 30% cut-off was arbitrarily chosen, also based on the original indications by Lewin,[3] presumably to assure that each component was quantitatively enough

to exert a significant biological and clinical effect on the natural history of the disease and on the patient's outcome. However, no systematic study has been performed, to date, to reaffirm the validity of this cut-off and, by contrast, the idea that even a minor component of high grade NEN can drive the patient's prognosis and should, therefore, be treated has been starting to dawn in the clinical setting.[10] In addition, the sole quantitative threshold, referring to an otherwise unspecified "neuroendocrine differentiation" appears to be a dangerous criterium, as one could tempted to use positive immunostains for general neuroendocrine markers as the prove of the neuroendocrine nature of the proliferation. This latter problem has been creating confusion in the literature and inconsistent terminology has been used.[11] The last WHO classifications of tumours of endocrine and digestive organs have clearly stated that, to define a MiNEN, the two components, neuroendocrine and non-neuroendocrine, are to be "morphologically recognizable".[8,9] This evolution in the definition transposes the need to clarify the clinico-pathological implications of diagnosing a mixed neoplasm, the behaviour of which can ultimately be predicted using the available knowledge on the outcome of its two components. Furthermore, it restores, in this field of pathology, the trust relationship between the pathologist and the oncologist, who find a common terminological ground again.

On this basis, we wonder whether the maintenance of the 30% cut of is still useful and/or essential in defining MiNENs, if the morphological diagnosis of a NEN, to be unquestionable, requires well established criteria, as it is. In other terms, as already stated by others, the "rule of 30%" is nearly intrinsic to the diagnosis of a MiNEN if we use strict morphological criteria to make the diagnosis.[1] Therefore, we believe that, especially in the case of mixed neoplasms including NEC, they should be incorporated in the concept of MiNEN, irrespective of the quantitative criteria.

Mixed neoplasms with an adenomatous component

As already mentioned, the WHO classification of digestive tumours does not recognize as MiNENs those neoplasms that include a preinvasive component, i.e. an adenoma.[9] This limitation is possibly justified by the negligible clinical impact of a preinvasive lesion, above all in terms of the therapeutic choice. We consider that this is a reasonable approach in the clinical practice, although it should not rule out the speculative importance of recognizing MiNENs composed of an adenomatous component and a NEN. Indeed, the comparative analysis of the neuroendocrine and non-neuroendocrine components has provided important clue to the pathogenesis of MiNENs and, in general of NENs, although most of the knowledge has been obtained in MiNENs composed of adenocarcinoma and NEC.[17, 18, 20, 21] Analogously, the recognition and the analysis of MiNENs composed of other possible combinations, including NETs and adenomatous lesions, may shed light on the mechanisms underlying their own development and, moreover, on the possible similarities or differences with the relative pure forms. We produced preliminary results on MiNENs composed of NET and adenoma, which we called MANET,[23] and of NET and adenocarcinoma,[22] demonstrating the monoclonal origin of the two components. However, this topic deserve further study on the specific alterations shared by the two components and on their roles in the pure forms of NET, adenoma and adenocarcinoma.

For these reasons, we believe that mixed neoplasms with an adenomatous component may, in principle, be considered as MiNENs and possibly diagnosed as such.

Neuroendocrine differentiation and MiNENs after neoadjuvant therapy

Due to the specific qualitative and quantitative criteria required to define a neoplasm as MiNEN,[9] it is clear that the definitive diagnosis can be exclusively performed on resected specimens and it can be only suspected on biopsy material, in the rare cases in which both

tumour components are present. Therefore, it is not surprising that an adenocarcinoma or squamous cell carcinoma diagnosed on a preoperatory biopsy turns out to be a MiNEN after the examination of the surgical sample. Conversely, it is less frequent that a MiNEN is identified after the preoperatory diagnosis of a NEN, usually a NEC, since the neuroendocrine component of MiNENs is often deeply located and is more difficult to be biopsied. In this context, the finding of a MiNEN in cases with a preoperatory diagnosis of adenocarcinoma then resected after neoadjuvant therapy has opened a large debate on the possible role of neoadjuvant therapy in inducing neuroendocrine differentiation or "MiNEN transformation" with its consequent impact on patient's outcome. This intriguing task merits specific considerations.

The real role of neoadjuvant therapy in stimulating neuroendocrine differentiation in digestive cancer, which may ultimately evolve in the morphological appearance of a MiNEN, is difficult to be established. Indeed, due to the aforementioned diagnostic limits of biopsy examination, several neoplasms considered as "post-therapy induced MiNENs" could be originally underdiagnosed MiNENs, the morphological features of which become evident only when an adequate sample (surgical specimen) is available for the histopathological examination. On the other hand, several studies demonstrated an increased number of neuroendocrine cells in digestive adenocarcinomas after preoperative neoadjuvant therapy, which was higher in patients receiving chemoradiotherapy than in those only receiving radiotherapy. [70-72] Although the presence of neuroendocrine differentiation in residual tumours after preoperative therapy was found to be associated with worse prognosis in oesophageal and gastroesophageal adenocarcinomas,[71] this was not observed in rectal adenocarcinomas.[73] This feature may have a practical clinical impact, so it needs to be better explored in future clinical investigations on larger series. Mechanisms underlying this

phenomenon are still not clear and two main hypotheses have been proposed.[70] The first evokes the resistance of pre-existing neuroendocrine cells to cytotoxic effects of neoadjuvant therapy, as they represent terminally differentiated nonproliferating cells low responsive to treatment effect, but this does not explain the worsening of prognosis. The second hypothesis suggests that cytotoxic injury itself stimulates neuroendocrine differentiation as observed in prostatic adenocarcinoma cell lines.[74,75] In addition, the neuroendocrine transformation has been also described in anti-EGFR-treated adenocarcinomas of the lung, also if the molecular mechanisms of this transition have not been clarified, yet.[76] In most cases the increased neuroendocrine cells are only identified using immunohistochemistry, appearing either as scattered cells, difficult to be detected morphologically, or as small clusters immediately adjacent to, or budding off, typical neoplastic glands, hard to be quantify and apparently not reaching the 30% of the tumour burden.[70] In other cases, amphicrine features have been demonstrated, since separate neuroendocrine tumour components were not found, and the neuroendocrine phenotype was observed in mucous-secreting cells.[72]

Taken together, these observations seem to suggest that post-therapy effects can increase the number of neuroendocrine cells rather than transform an adenocarcinoma or a squamous cell carcinoma into a MiNEN. For this reason, in the last WHO classification of digestive NENs, carcinomas previously treated with neoadjuvant therapy showing a neuroendocrine component are not considered MiNENs, unless the diagnosis of MiNEN is established on pretreatment specimens.[9]

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Figure legends

Figure 1: Adenocarcinoma of the sigmoid colon with usual tubulo-papillary architecture (A) showing diffuse positive immunostaining for synaptophysin (B). Chromogranin A is completely negative (C).

Figure 2: Amphicrine carcinoma of the rectum with solid and glandular architecture composed of cells with amphophilic cytoplasm (A), which, at ultrastructural analysis, shows hybrid features with exocrine (apical microvilli, tight junctions, mucous granules) and neuroendocrine (dense core granules) features (B). Synaptophysin (C) and chromogranin A (D) are diffusely expressed.

Figure 3: Proposed pathogenetic pathways for MiNENs of large intestine. After a preclinical phase in which the neoplastic cell undergoes genetic events affecting the APC/beta-catenin pathway (driving to chromosomal instability) or the Wnt signaling pathway (driving to microsatellite instability), subsequent events lead to the development of adenocarcinomatous (pure adenocarcinoma), or poorly differentiated neuroendocrine (pure NEC) morphology, or both (MiNEN).[17-21]

Figure 4. Colonic MiNEN composed of adenocarcinoma and large cell NEC (A). The adenocarcinomatous component is well evident on the left, while the neuroendocrine component (right) shows a solid architecture and is immunoreactive for synaptophysin (B).

Figure 5. Colonic MiNEN composed of adenocarcinoma and NET (A). The NET component is positive for chromogranin A (B) and shows a low Ki67 proliferative index (C).

Figure 6. Mixed ductal neuroendocrine carcinoma of the pancreas showing neoplastic glands associated with a more solid component (A), which is positive for chromogranin A (B).

Figure 7. Example of a pancreatic ductal adenocarcinoma with entrapped islets associated with chronic pancreatitis. Islets show an ovoid shape with regular contours and appear

increased in number and size (A). Immunohistochemical stainings demonstrate that positive cells for insulin (B), glucagon (C), and somatostatin (D) are present in the islet and show the well-known intra-insular distribution.

Figure 8. PanNETs with entrapped ductules may represent a diagnostic challenge, especially in presence of a fibrous stroma (A). In other cases, small ductules are scattered among neuroendocrine tumour cells (B). Entrapped ductules lack cells atypia and show a regular shape. Immunohistochemistry for CK7 is useful to identify the regular distribution of ductules inside the tumour (C). Nuclear Ki67 expression is restricted to scattered ductular cells (D).

Figure 9. Mixed acinar-neuroendocrine carcinoma showing morphologically distinguishable solid acinar (left) and trabecular neuroendocrine (right) components (A). The neuroendocrine component is strongly positive for chromogranin A (B), while is negative for trypsin that is positive in the acinar component (C).

Figure 10: Diagnostic algorithm for the discrimination among MiNEN, carcinoma with neuroendocrine markers expression, and amphicrine carcinoma. *: synaptophysin, chromogranin A, INSM1; NE: neuroendocrine; SCC: squamous cell carcinoma; ADC: adenocarcinoma; DCC: ductal cell carcinoma; ACC: acinar cell carcinoma

Table 1. Prognostic classification and distribution of digestive MINENs

High grade malignant

Mixed adenocarcinoma-NEC^
- Distal oesophagus
- Gastroesophageal junction
- Stomach
- Small intestine
- Appendix
- Large intestine
- Gallbladder

Mixed squamous cell carcinoma-NEC
- Oesophagus
- Large bowel
- Anal canal

Mixed ductal adenocarcinoma-NEC
- Pancreas

Mixed acinar cell carcinoma-NEC
- Pancreas

Mixed ductal adenocarcinoma-acinar cell carcinoma-NEC
- Pancreas

Mixed cholangiocarcinoma-NEC
- Liver

Mixed ductal adenocarcinoma-NET*
- Pancreas

Mixed acinar cell carcinoma-NET*
- Pancreas

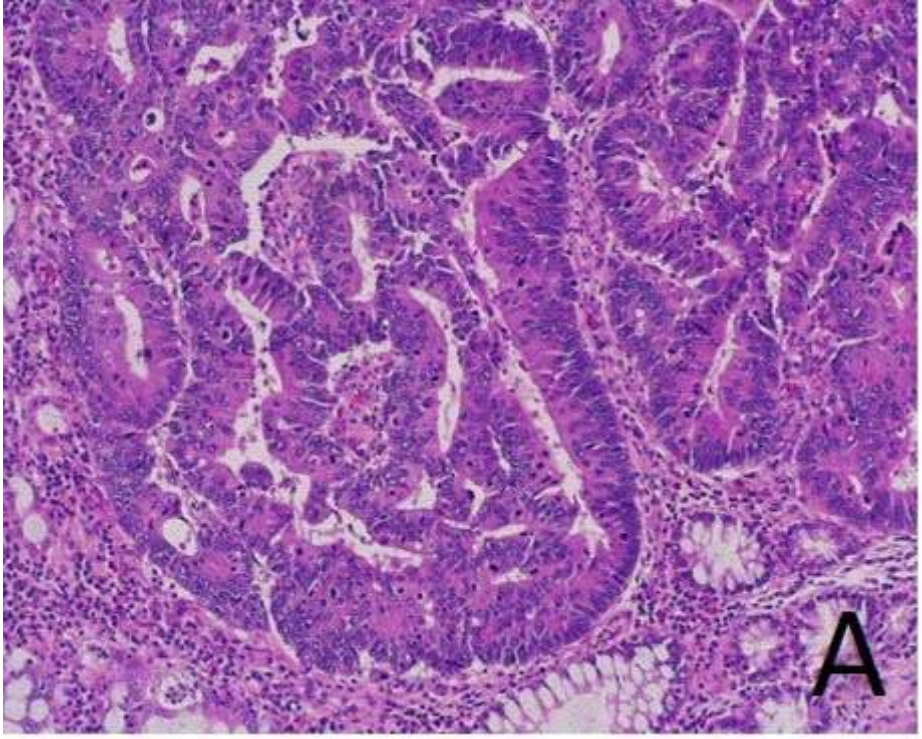
"Intermediate" grade malignant

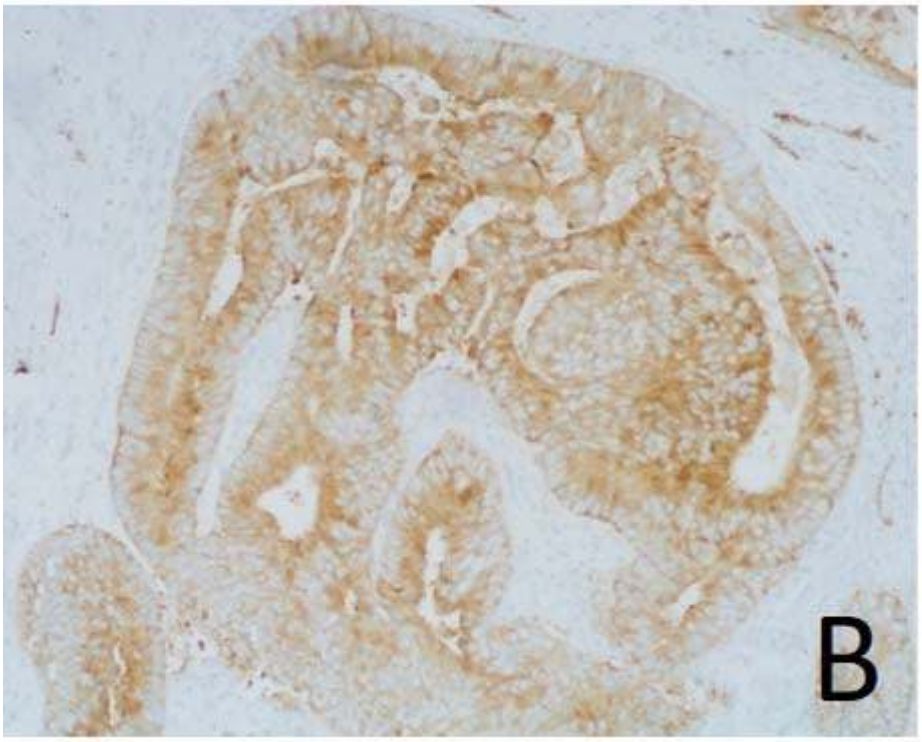
Mixed adenocarcinoma-NET*
- Gastroesophageal junction
- Stomach
- Duodenum
- Large intestine

Low grade malignant°

Mixed adenoma-NET (MANET)
- Stomach
- Duodenum
- Large intestine

NEC: poorly differentiated neuroendocrine carcinoma; ^: for this subtype the term mixed adenoneuroendocrine carcinoma (MANEC) can be retained; *: grade according to WHO classification[9]; NET: neuroendocrine tumour; °: by the WHO definition these cases are not formally included in the MiNEN category[9].


A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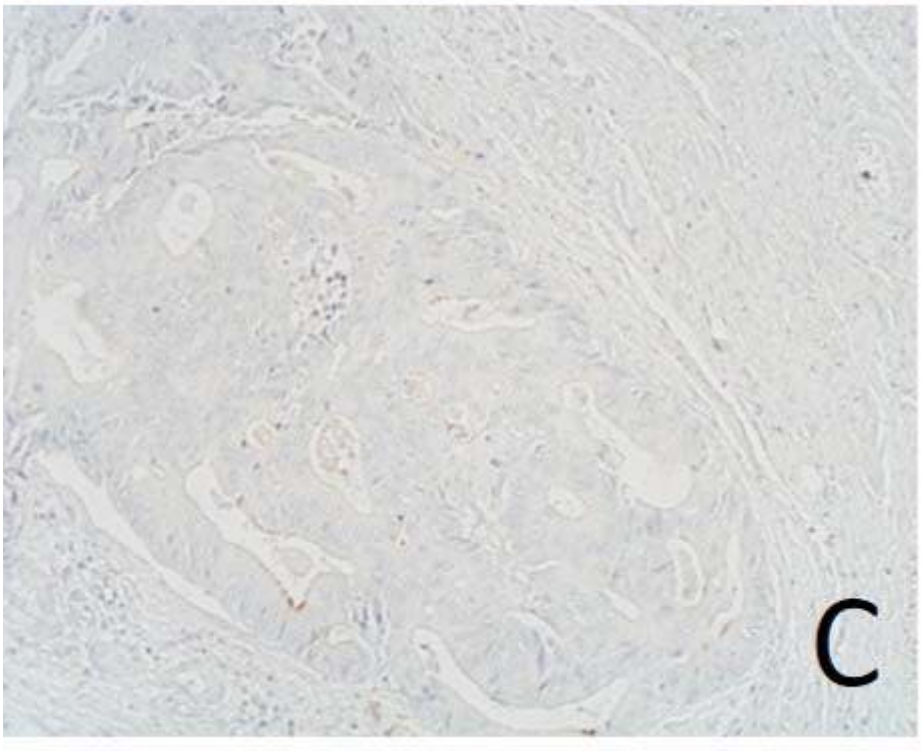
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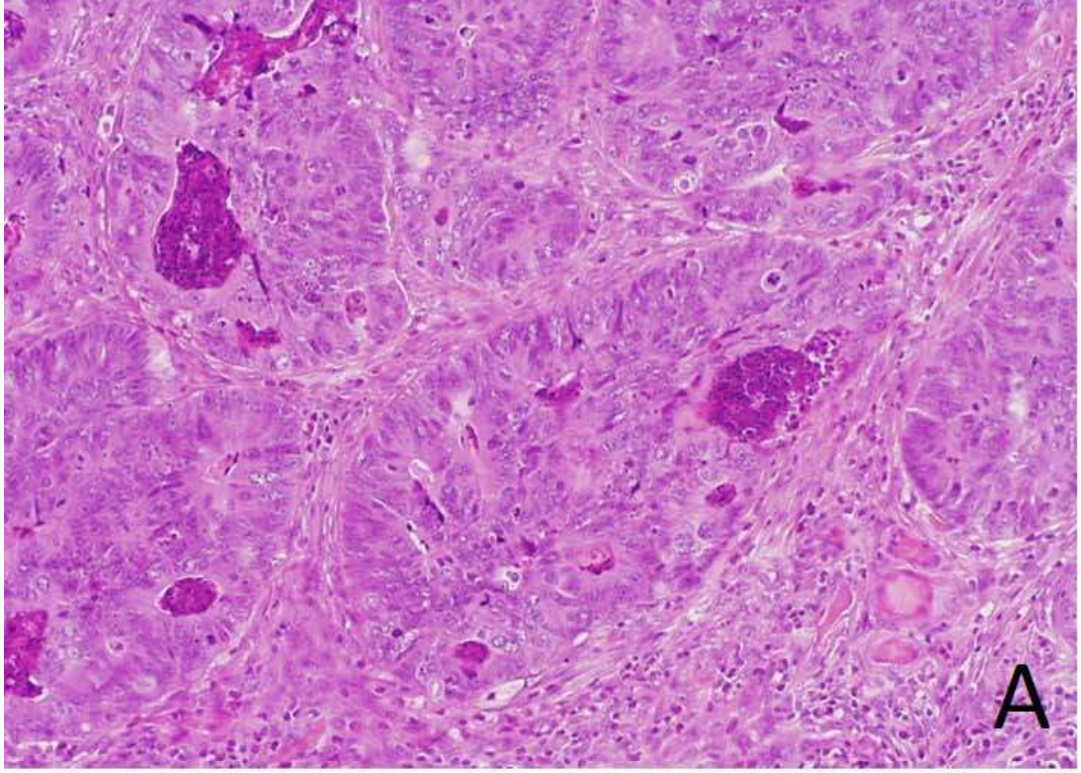
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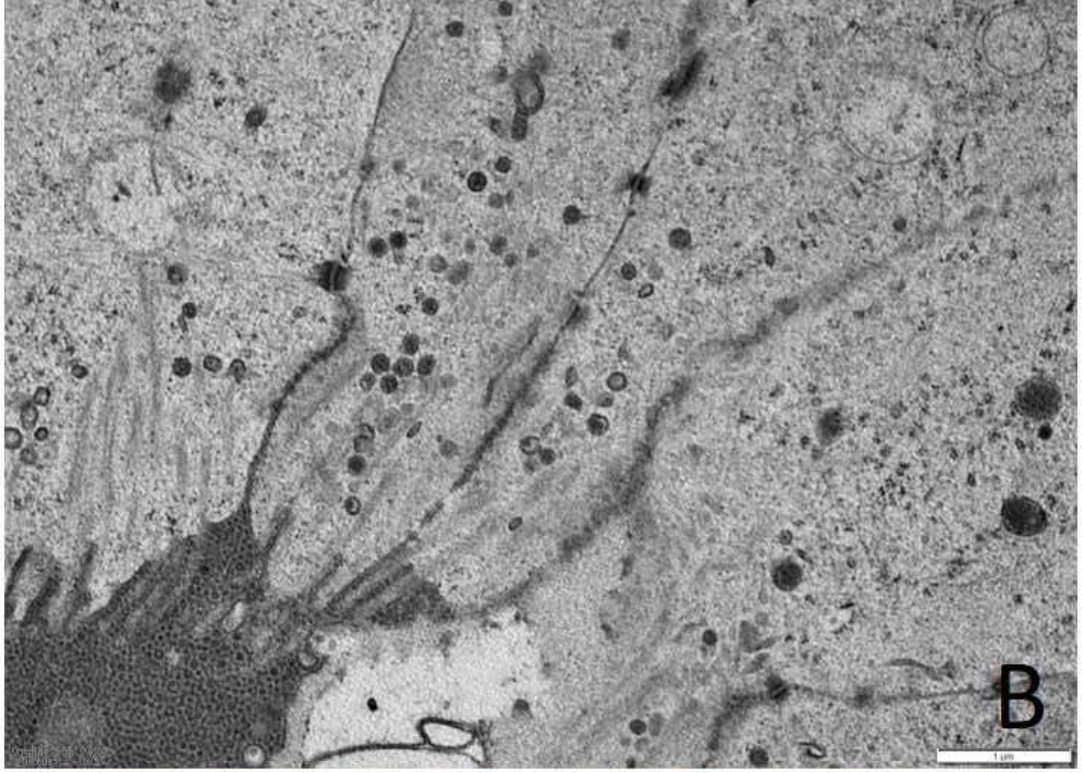
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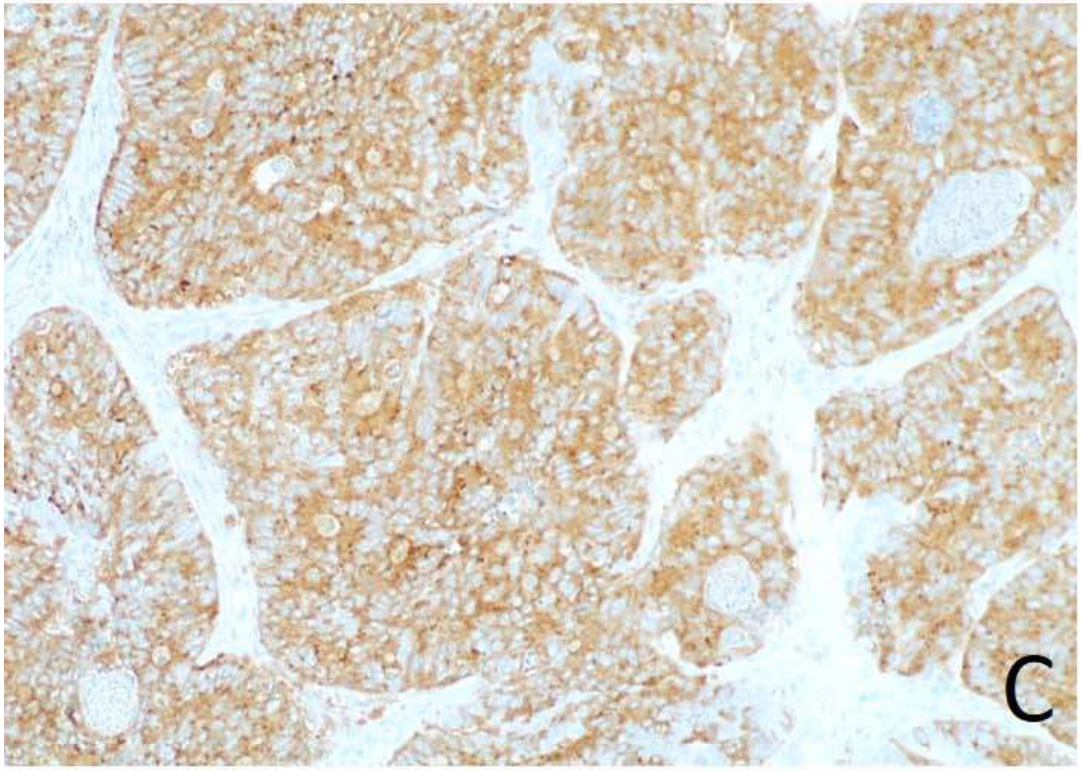
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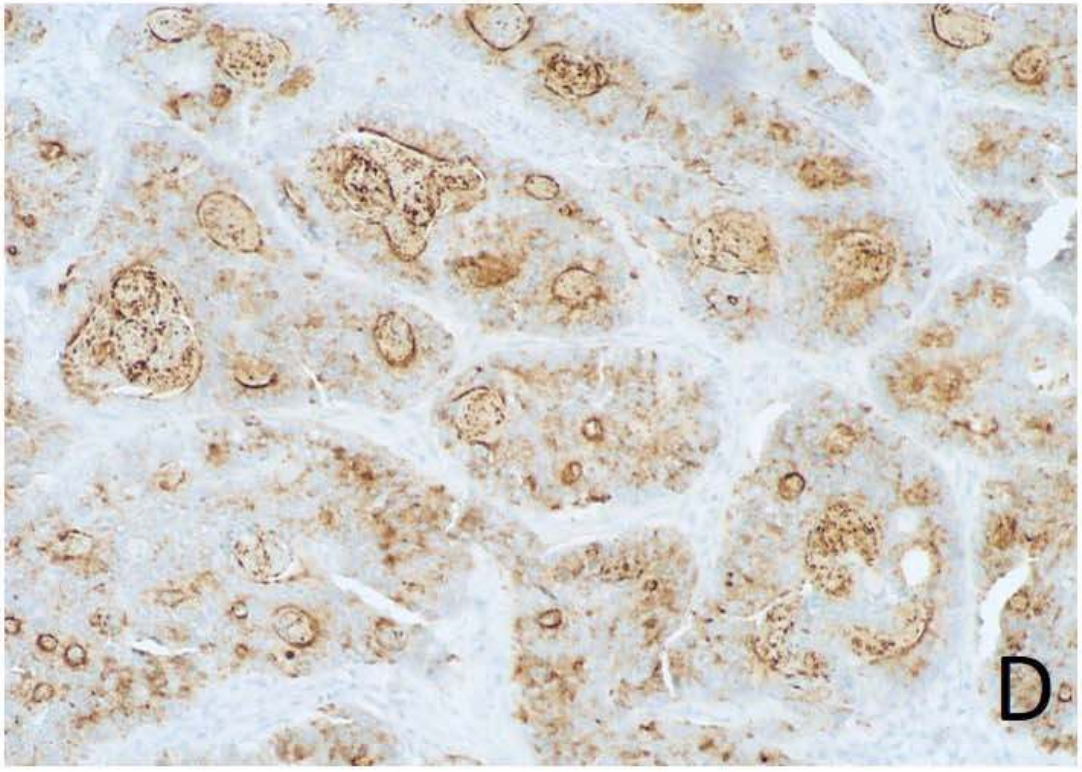
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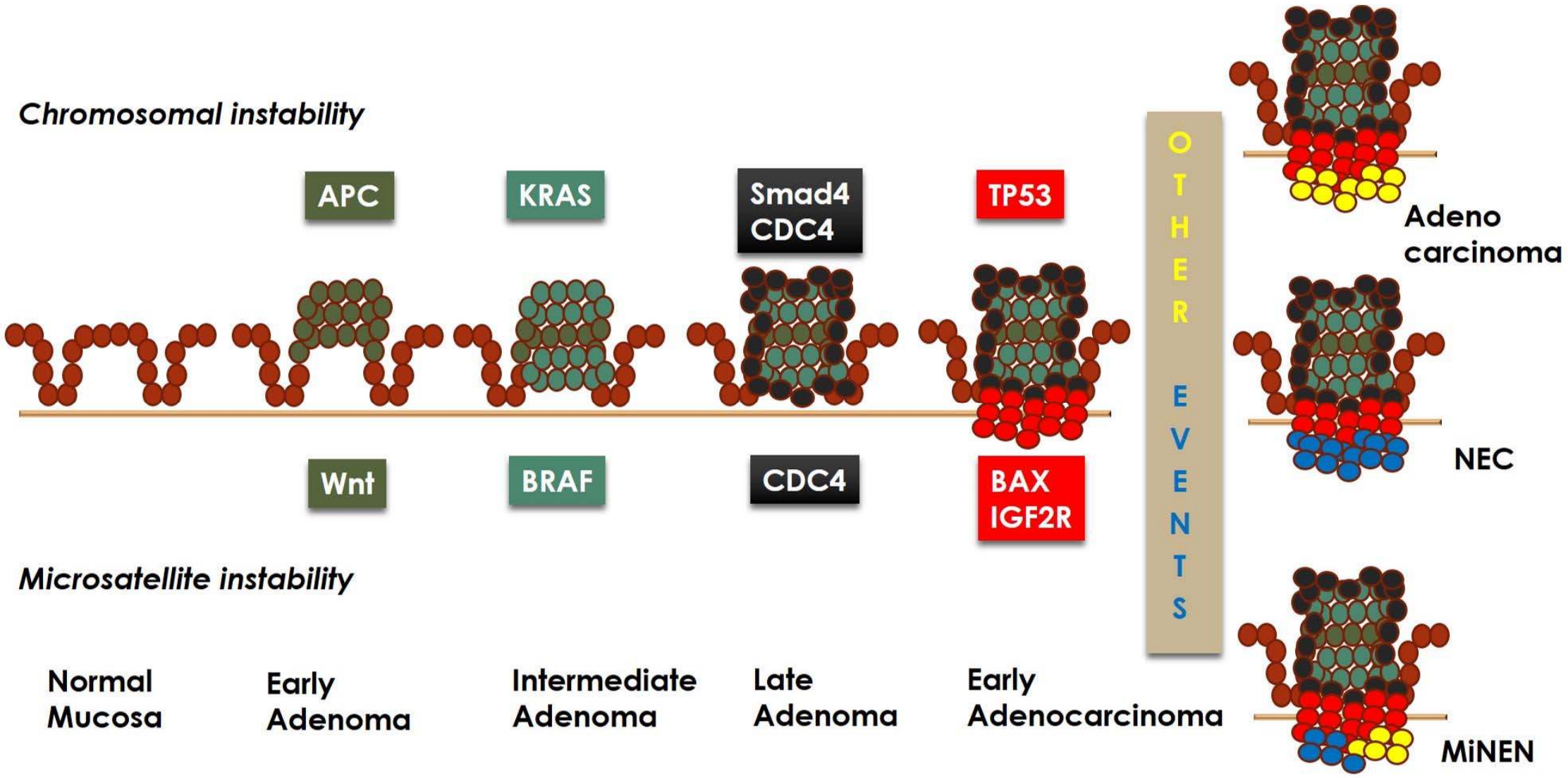
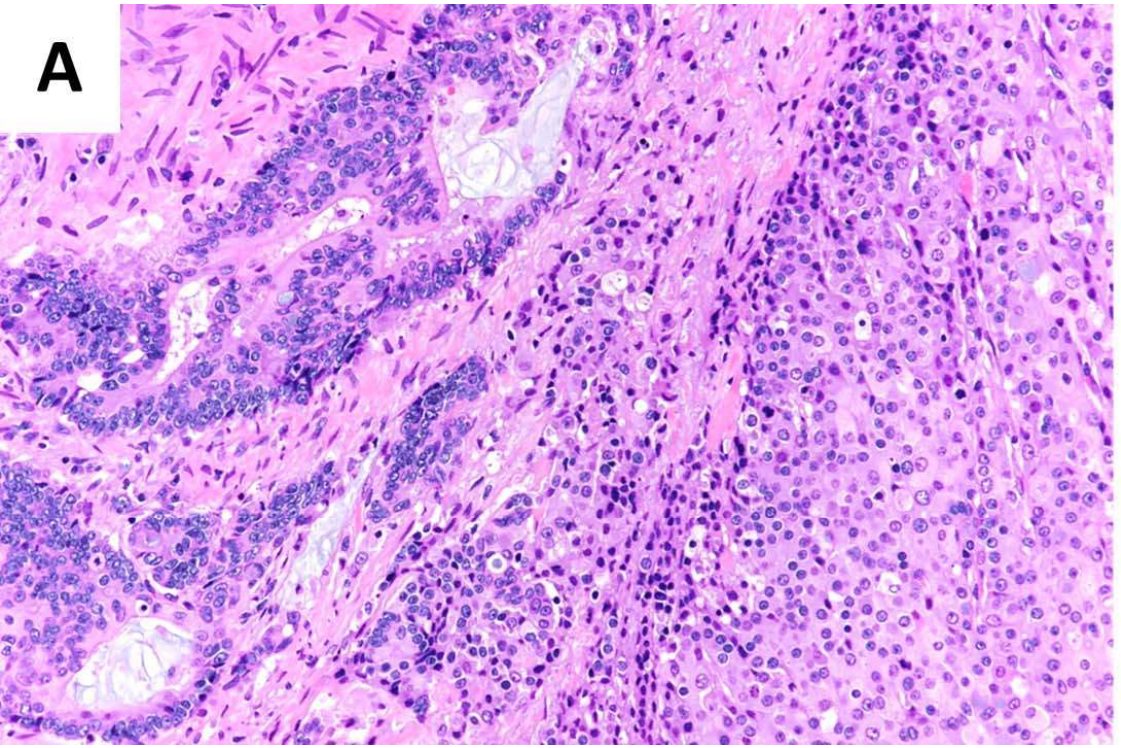
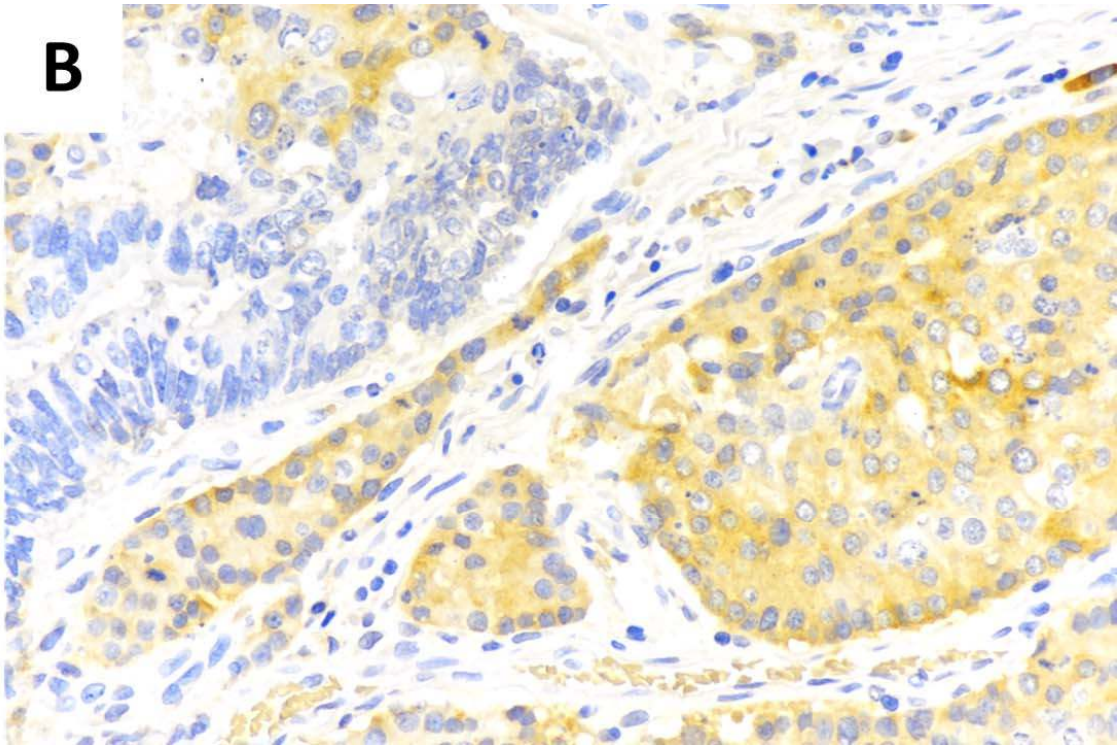

Chromosomal instability
APC
KRAS
Smad4
CDC4
TP53
OTHER EVENTS
Adeno carcinoma
NEC
MiNEN
Wnt
BRAF
CDC4
BAX
IGF2R
Microsatellite instability
Normal Mucosa
Early Adenoma
Intermediate Adenoma
Late Adenoma
Early Adenocarcinoma


A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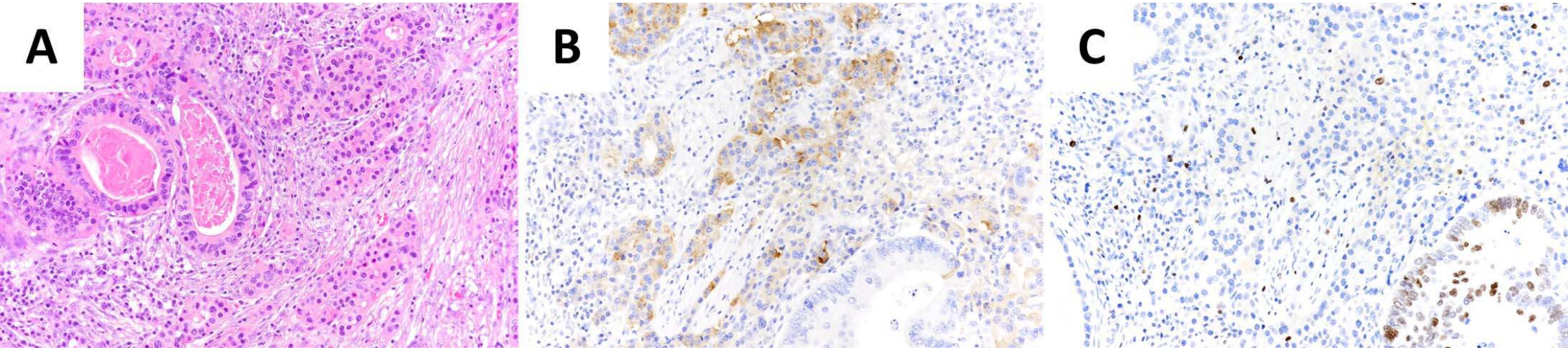
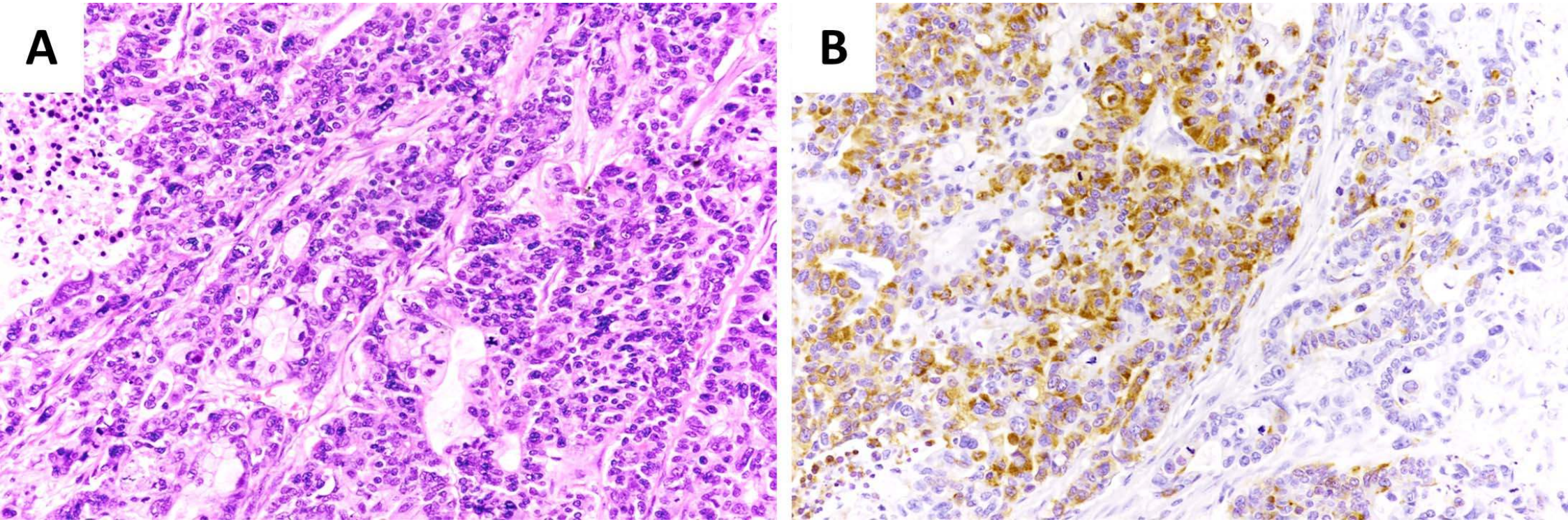
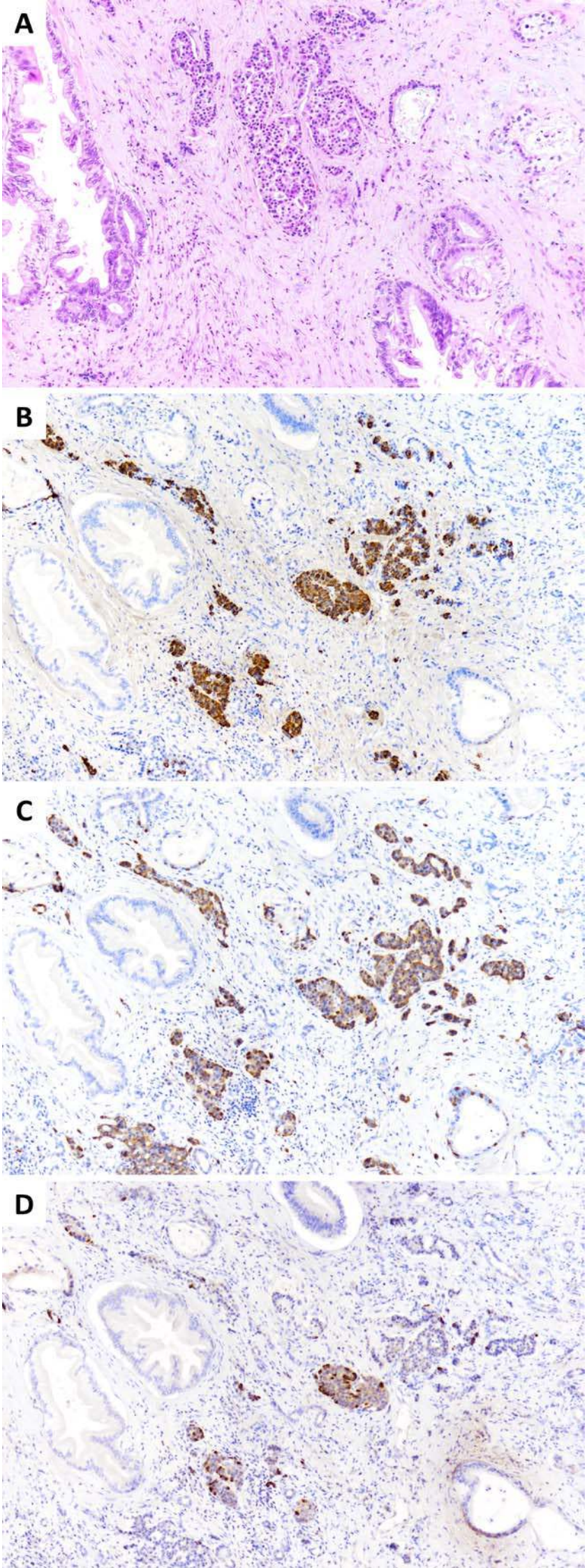
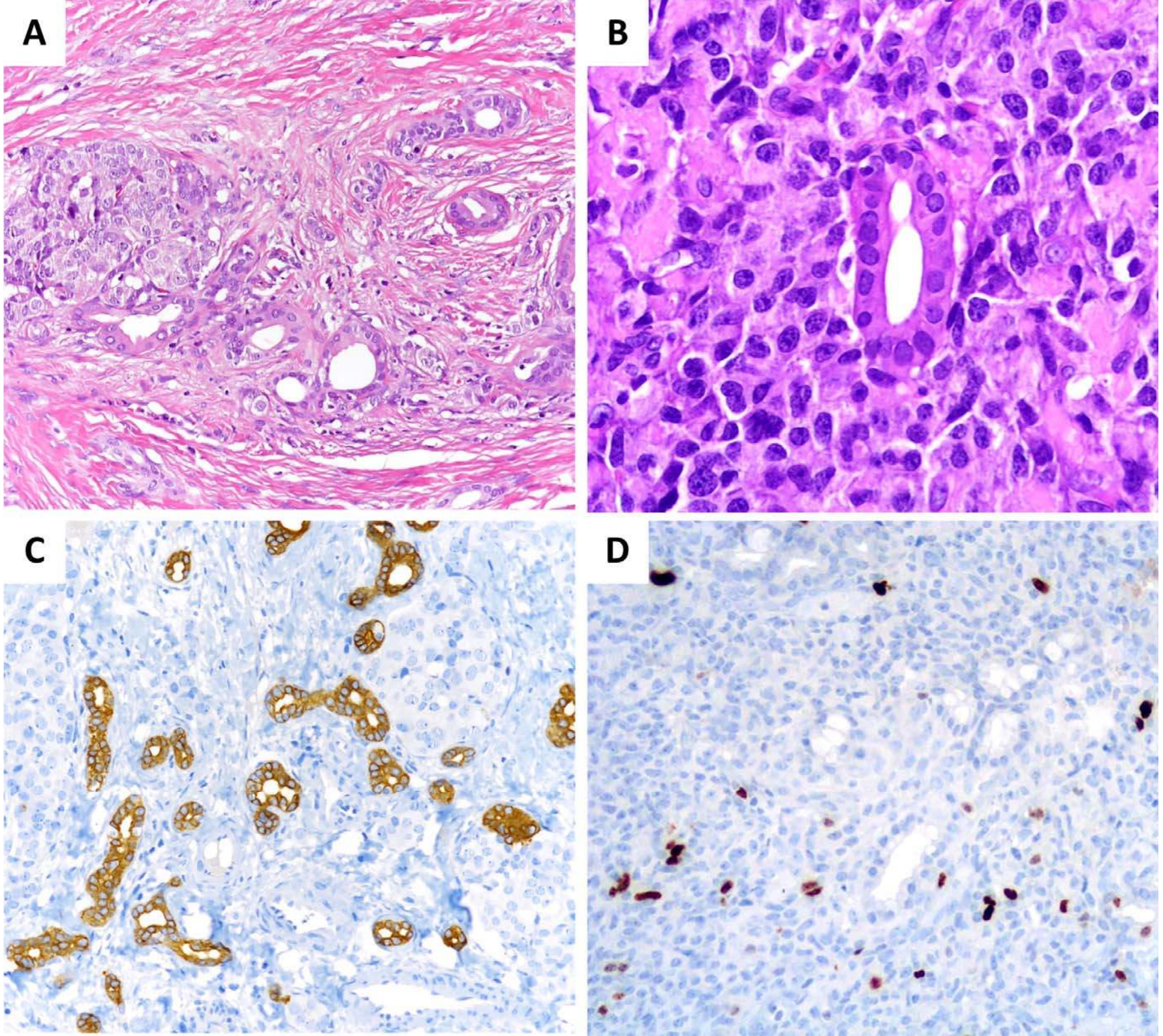

A
B
C


A
B


A
B
C
D


A
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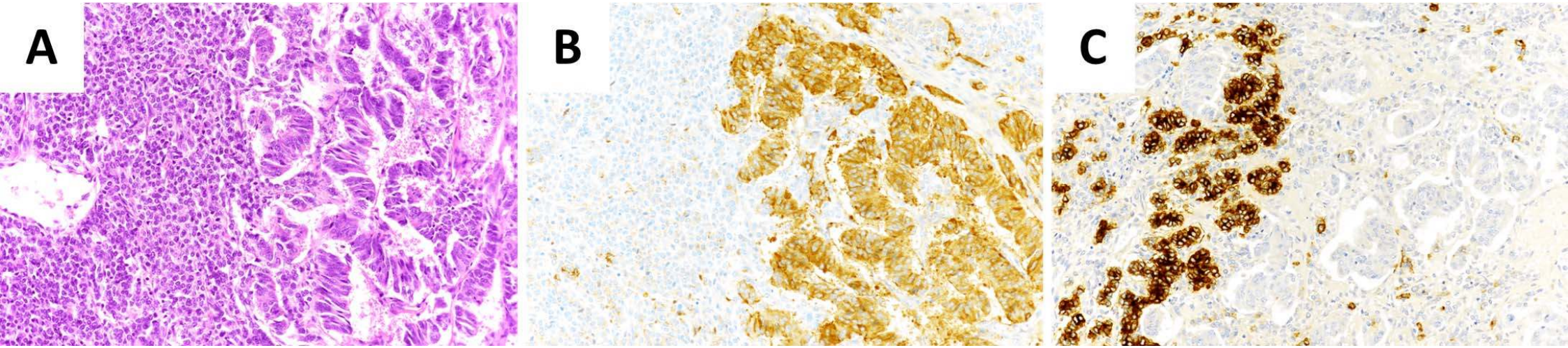
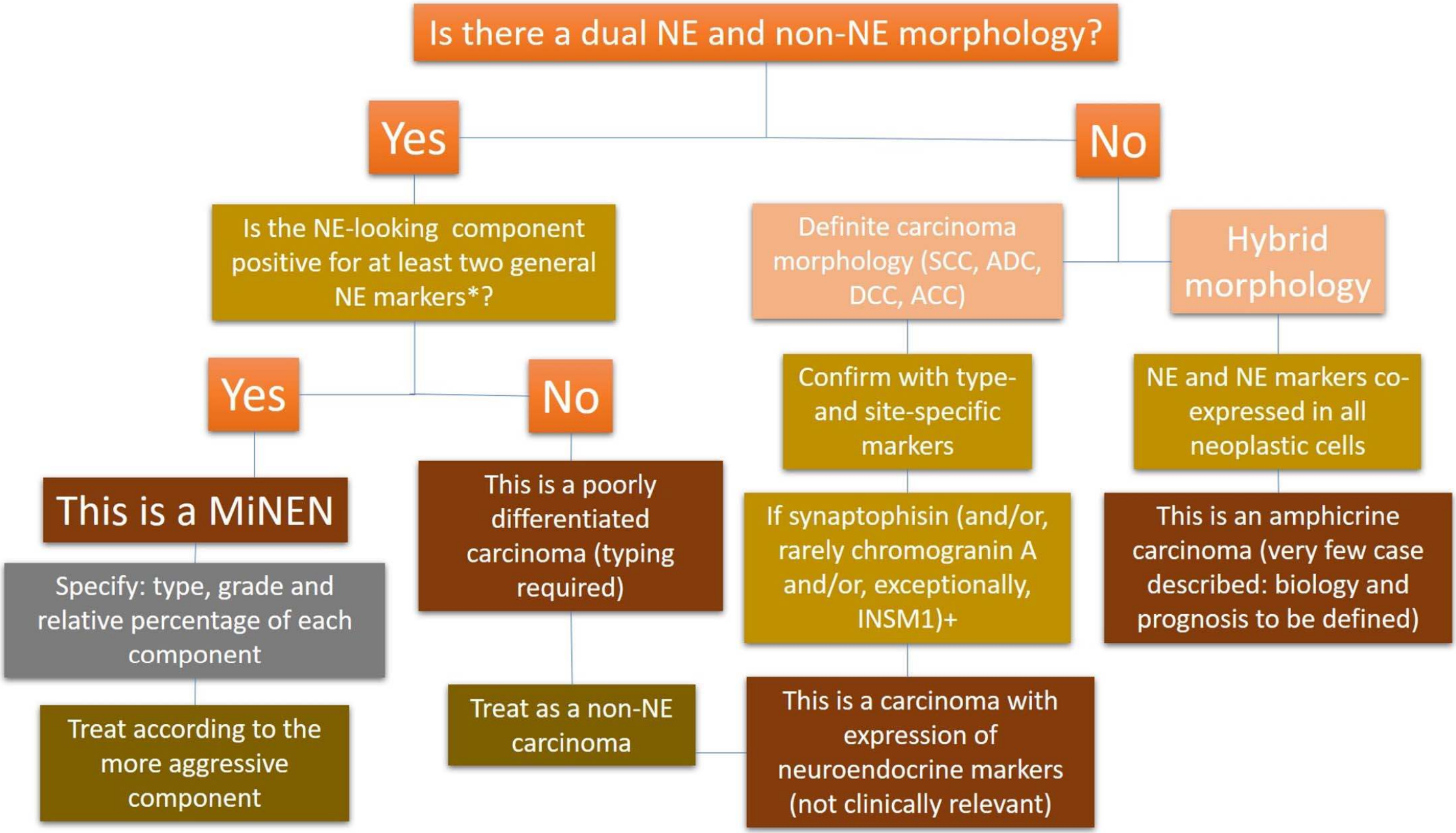

A
B
C


Is there a dual NE and non-NE morphology?
Yes
No
Is the NE-looking component positive for at least two general NE markers*?
Definite carcinoma morphology (SCC, ADC, DCC, ACC)
Hybrid morphology
Yes
No
Confirm with type- and site-specific markers
NE and NE markers co-expressed in all neoplastic cells
This is a MiNEN
This is a poorly differentiated carcinoma (typing required)
If synaptophisin (and/or, rarely chromogranin A and/or, exceptionally, INSM1)+
This is an amphicrine carcinoma (very few case described: biology and prognosis to be defined)
Specify: type, grade and relative percentage of each component
Treat as a non-NE carcinoma
This is a carcinoma with expression of neuroendocrine markers (not clinically relevant)
Treat according to the more aggressive component