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Multilayered heterogeneity as an intrinsic hallmark of neuroendocrine tumors

Sergio Pedraza-Arévalo [1,2,3,4,5] · Manuel D. Gahete [1,2,3,4,5] · Emilia Alors-Pérez [1,2,3,4,5] · Raúl M. Luque [1,2,3,4,5] · Justo P. Castaño [1,2,3,4,5]

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Abstract

Neuroendocrine tumors (NETs) comprise a complex and highly heterogeneous group of neoplasms that can arise all over the body, originating from neuroendocrine cells. NETs are characterized by a general lack of symptoms until they are in advanced phase, and early biomarkers are not as available and useful as required. Heterogeneity is an intrinsic, pivotal feature of NETs that derives from diverse causes and ultimately shapes tumor fate. The different layers that conform NET heterogeneity include a wide range of distinct characteristics, from the mere location of the tumor to its clinical and functional features, and from its cellular properties, to the core signaling and (epi)genetic components defining the molecular signature of the tumor. The importance of this heterogeneity resides in that it translates into a high variability among tumors and, hence, patients, which hinders a more precise diagnosis and prognosis and more efficacious treatment of these diseases. In this review, we highlight the significance of this heterogeneity as an intrinsic hallmark of NETs, its repercussion on clinical approaches and tumor management, and some of the possible factors associated to such heterogeneity, including epigenetic and genetic elements, post-transcriptional regulation, or splicing alterations. Notwithstanding, heterogeneity can also represent a valuable and actionable feature, towards improving medical approaches based on personalized medicine. We conclude that NETs can no longer be viewed as a single disease entity and that their diagnosis, prognosis and treatment must reflect and incorporate this heterogeneity.

Keywords Neuroendocrine tumors · Heterogeneity · Hallmark · Cancer · Carcinoid · Complexity

1 Introduction

Neuroendocrine tumors (NETs) are a highly heterogeneous group of neoplasms arising from the cells of the neuroendocrine system, which are widely distributed throughout the body. These tumors have been classically known as carcinoids, a term used for the first time in 1907 by Siegfried Oberndorfer [1]. NETs originate from cells with a marked duality, for they may simultaneously exhibit the typical characteristics of both neural and endocrine cells. Thus, owing to their neuroendocrine nature, NET cells can be affected by neural modulators, and might (over)secrete diverse peptide hormones and biogenic amines, such as chromogranin A (CgA) or synaptophysin, which can consequently be used as biomarkers, and could lead to syndromic comorbidities [2, 3]. However, a high proportion of NETs progresses without obvious clinical symptoms, which represents one of the most important problems associated to these pathologies. Recent improvements in diagnostic techniques have revealed that the incidence of NETs is higher than previously considered [4] and, even worse, SEER data indicate that the general incidence of NETs, as well as the incidence of mostly every different type of NETs, is increasing over the last years, particularly those from pancreas, lung and small intestine [5]. Therefore, a more profound and detailed characterization of these pathologies seems necessary for the appropriate management of NETs patients.

✉ Raúl M. Luque
raul.luque@uco.es

✉ Justo P. Castaño
justo@uco.es

1 Maimonides Institute for Biomedical Research of Cordoba (IMIBIC), Avenida Menéndez Pidal s/n, Edificio IMIBIC, 14004 Córdoba, Spain

2 Department of Cell Biology, Physiology, and Immunology, Universidad de Córdoba, Córdoba, Spain

3 Reina Sofia University Hospital, Córdoba, Spain

4 CIBER Fisiopatología de la Obesidad y Nutrición (CIBERobn), Córdoba, Spain

5 Agrifood Campus of International Excellence (ceiA3), Córdoba, Spain

In this scenario, one of the most recognizable hallmarks of NETs is their extraordinary heterogeneity, which profoundly influences the diagnosis, prognosis and medical response of these neoplasms, and severely hampers the research to identify novel and more general biomarkers and/or therapeutic targets. Indeed, the heterogeneity of NETs has been recognized from the first definitions and classifications of NETs established by Williams and Sandler in 1963 [6] and, since then, it has been consistently and tightly linked to this disease. In fact, heterogeneity seems to belong to the intrinsic nature of NETs and would stem from (and consist of) several superimposed factors, ranging from tumor location or cell type from which the tumor derives, to its functionality, associated clinical parameters, etc., as it will be discussed in this review. Nevertheless, despite the drastic limitation that heterogeneity imposes in the identification of more general diagnostic biomarkers or medical treatments for NETs, it seems conceivable that this particularity could be converted into a valuable tool to better classify and discriminate the different types of NETs and, eventually, as an actionable intrinsic feature enabling a more personalized management of the patients.

2 Levels of heterogeneity in NETs

The heterogeneity of NETs is not attributable to a single factor, but to a sum of elements that reside in different levels, some of them of very distinct and apparently distant nature (e.g. tumor location vs. secretory function), but that are nonetheless interconnected, and can be conceptually visualized as a multilayered set of variable features (as illustrated in Fig. 1). Accordingly, NET heterogeneity should be analyzed from several points of view (i.e. the levels of heterogeneity), which closely correspond to different tumor features. Thence, these levels of heterogeneity would need to be integrated and modeled in order to better understand NETs, which could help to develop more precise avenues to tailor personalized medicine for NET patients.

2.1 Location

In 1963, Williams and Sandler [6] proposed the first classification for NETs, attending to the embryonic origin of the transformed cells and, therefore, to their location. This classification is still, in fact, currently used, and subdivides NETs according to their location in foregut (lungs, thymus, esophagus, stomach, duodenum and pancreas), midgut (jejunum, ileum and cecum) and hindgut NETs (distal colon and rectum). Although this classification is not sufficient to define the NETs and to provide substantial clinical information, it is still remarkably useful, inasmuch as the location of the tumor determines other additional tumor characteristics such as the range of cell types potentially originating it or the molecular mechanisms underlying their development and/or progression.

2.1.1 Foregut NETs

Foregut tumors develop in the respiratory tract, thymus, stomach, duodenum and pancreas. In particular, *lung* NETs are among the most common NETs. Low grade lung NETs comprise approximately the 27% of all NETs, with 0.2–2 cases per 100,000 habitants in USA and Europe [7]. These tumors arise from either individual or clusters of pulmonary neuroendocrine cells. Lung NETs represent one of the main NET types by incidence and can be subdivided in four types depending on the histology and in three groups regarding their grade: low grade typical carcinoids (TC), intermediate grade atypical carcinoids (AC) and high grade large cell neuroendocrine carcinoma (LCNEC) and small cell lung carcinoma (SCLC) [7–9].

NETs from the *thymus* are uncommon and may appear with an ample spectrum of aggressiveness, from well differentiated to poorly differentiated tumors. They usually appear associated with other pathologies, such as Cushing's or MEN1–2 syndromes [10–12]. Thymic NETs differ from thymic adenocarcinomas in the presence of neuroendocrine markers and special architectural characteristics, which are crucial features that determine the different development of both types of tumors. The classification used is the same than that presented for lung NETs [10, 11, 13, 14].

In the case of NETs from the *pancreas*, also called PanNETs or PNETs, are usually solitary, between 1 and 5 cm, well differentiated and sporadic tumors, but they may be also associated with other disorders as MEN1, VHL or NF1 syndromes. These tumors arise from the islets of Langerhans and can exhibit different features depending on the cell type of origin [15–18]. NETs of the *esophagus* are very rare and aggressive tumors, but they are reasonably sensitive to treatment. These esophagus NETs usually appear as large ulcerated and poorly differentiated neoplasm at the third region of the organ and may also comprise exocrine gland derived components [15, 19]. The so-called gastric NETs appear in the *stomach* and are commonly classified in three different groups, depending on their histology and the clinical features. Type I tumors (70–80%) are composed of enterochromaffin-like (ECL) cells and are commonly linked with chronic atrophic gastritis; type II tumors (5–6%) are associated with MEN1 and Zollinger-Ellison syndrome and also originate from ECL cells; and type III tumors (10–15%) are independent of gastrin secretion and not associated with ECL hyperplasia. Types I and II are commonly multiple small tumors, found in fundus or corpus, while type III tumors are larger, typically single and found in any region [20, 21]. Duodenal NETs are mostly found in the first and second part of *duodenum*, with a great accumulation near the ampulla of Vater. They are usually small, solitary and non-functional [20]. NETs from esophagus, stomach, gut and pancreas have been unified under the term gastroenteropancreatic NETs

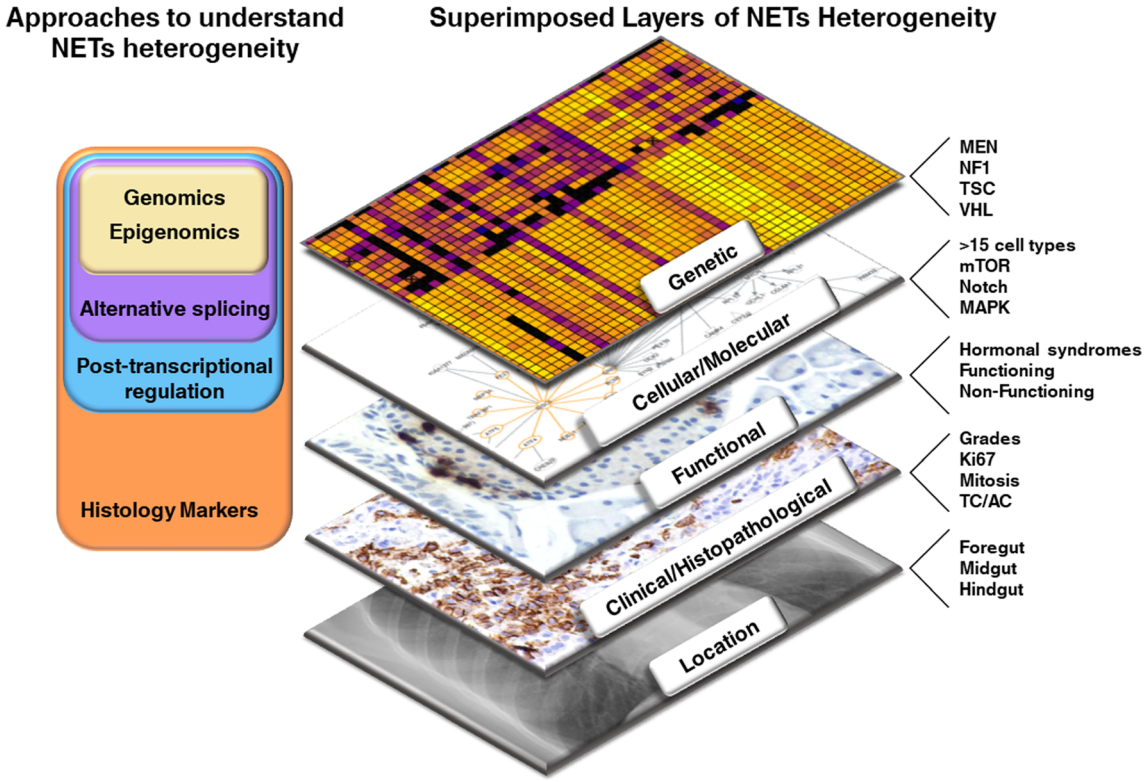

Fig. 1 Levels of heterogeneity in Neuroendocrine Tumors. This figure represents the different layers of this heterogeneity interrelated and affecting each other, which results in tumors with high complexity and variability. In addition, the left panel underscores some of the different approaches that are emerging as novel tools to understand each layer of NETs heterogeneity

(GEP-NET) in order to designate a group of NETs that share common features and similar origin [22–24]. GEP-NETs show an incidence of 2,5–5 new cases per 100,000 habitants in USA [25].

2.1.2 Midgut NETs

Midgut NETs encompass NETs from the *jejunum* and *upper ileum*, which are usually well differentiated and do not commonly present symptoms [26]; in rare cases, these NETs can secrete hormones such as ACTH [27]. In the case of *distal ileum* NETs, the majority of them are originated near to the ileocecal valve and are not usually associated with inherited syndromes. These tumors are usually immersed into sclerotic stroma, which can obstruct the bowel [15]. The *appendix*, which sits at the junction of the small intestine and large intestine, can develop midgut and hindgut NETs [28]. These tumors are most commonly found in the tip, presenting as a well differentiated, 1–2 cm tumoral mass, and, in some cases, infiltrating the wall of the organ [15]. In this line, it is also worth noting the existence of goblet cell carcinomas of the appendix, a group of heterogeneous neoplasms that exhibit both glandular and neuroendocrine features [29].

2.1.3 Hindgut NETs

As for the *large intestine*, NETs are usually poorly differentiated and tend to appear more frequently in the rectum. They are commonly small and may appear along with other colorectal carcinomas [15, 30, 31]. Interestingly, NETs may rarely appear in the *presacral region*, between the rectum and the sacrum, but when present therein, they are normally well differentiated, associated with gut cysts or other pathologies [15, 32, 33].

2.1.4 Other NETs

Medullary *thyroid* carcinoma (MTC) is the typical form of NET observed in the thyroid. MTCs arise from the C-cells and exhibit a notable heterogeneity, from indolent well-differentiated tumors to aggressive malignant tumors, with high mortality. The majority are sporadic tumors, about 25% of MTC are hereditary and appear in the context of MEN2 syndrome or familial MTC [34–37]. In addition, it has been a matter of debate for a long time if thyroid NETs arising from small cells really exist or they are just lymphomas. In this regard, there is currently a consensus for the existence of small cell thyroid NETs [38]. Remarkably, NETs (i.e. paraganglioma) can also appear in the *parathyroid* gland [39–41], but their incidence is particularly reduced.

Paragangliomas and pheochromocytomas are NETs that arise from *autonomic paraganglia* and *adrenal medulla*, respectively. In fact, WHO defines pheochromocytomas as an intra-adrenal paraganglioma, emphasizing their common origin from neuroectoderm, and both may appear in patients with the same genetic predispositions. Approximately, 40% of the cases are linked to a germline mutation. About 25% of cases present metastasis, but they are mostly poorly aggressive NETs with an associated high morbidity and mortality caused by hormonal syndromes. The majority of paragangliomas appear in the parasympathetic tissues of head and neck [42–47].

Finally, it should be noted that although the locations described above are the most frequent for NETs, they could develop in virtually any tissue containing neuroendocrine cells. This is the case of NETs present in ureter [48], bladder [49], prostate [50], ovary [51, 52], cervix [53, 54], breast [55], skin [56], testis [57], kidney [58], sublingual gland [59], gallbladder [60] or even sinonasal tract [61]. This basic, concise enumeration of the site-dependent catalog of NETs highlights the unusually wide variety of locations where these tumors can arise throughout the body, and, as well, provides the first source of heterogeneity, in that the organ/tissue of origin already imprints a distinct set of influencing features defining the precise nature of the tumor.

2.2 Histopathological features

NETs are also greatly heterogeneous from the histopathological point of view. Consequently, the evaluation, definition and establishment of common criteria to define the histotype and grade of NETs have been controversial. Actually, since the terminology and clinical criteria used to define NETs differs when two similar NETs arise at different locations, several tumor classification systems have been proposed. In addition, in that pulmonary and GEP-NETs are the most predominant subtypes, the majority of proposed classification systems have been centered on these particular NET subtypes [4, 62].

Specifically, regarding GEP-NETs, the WHO Classification of Tumors of 2010 specified that the NET term comprises well and moderately differentiated, low and intermediate grade tumors, while the term neuroendocrine carcinoma (NEC) should stand for poorly differentiated and high-grade lesions. However, the term neuroendocrine neoplasm (NEN) should be used to comprise all neuroendocrine tumors and carcinomas [25]. In this version of the classification, the WHO included the proliferation rate (Ki-67 index), proposed by the European Neuroendocrine Tumor Society (ENETS) and following the recommendation of the International Union Against Cancer (IUAC) and the American Joint Cancer Committee (AJCC), as well as the histopathological criteria to classify the NETs. The result was a classification aimed to standardize the grouping of the tumors in three grades, as follows: Grade 1 NET, mitotic count < 2, Ki-67 ≤ 2%; Grade 2 NET, mitotic count 2–20, Ki-67 3–20%; and Grade 3 NEC, mitotic count > 20, Ki-67 > 20 [15, 25, 62]. Nevertheless, WHO complemented this classification in 2017, including the Grade 3 NET, which present well-differentiated morphology and mitotic indexes higher than 20%, and Mixed Neuroendocrine-Non-Neuroendocrine Neoplasms (MiNEN), combinations of neuroendocrine component, usually poorly differentiated, and a non-neuroendocrine component, generally adenocarcinomas [63, 64].

Additionally, in 2015, the WHO, supported by other organisms, expanded their original classification by including lung and other similar NETs, such as those from thymus. Whereas previous classifications were based on light microscopy evaluations and, only in some cases, immunohistochemistry determinations, the new classification was more complex and precise. Thus, new criteria and terminology for the diagnosis using small biopsies and cytology have been included, as these techniques are the most commonly used in these pathologies, except for LCNEC, only diagnosed by resection. Particularly, this novel classification includes the differentiation between SCLC, LCNEC and carcinoids (TC and AC) for lung NETs, which is different to other schemes or classifications that apply a concept of tumor grading, similar to GEP-NETs. However, this new proposal does not present clinical advantages compared to the previous WHO classification. Specifically, SCLC and LCNEC are included in a unique high-grade group, which is not supported by any causative relationship with carcinoids, but present severe differences compared to them, such as higher mitotic rate (which poses a main role of Ki-67 in these pathologies), necrosis and genetic abnormalities. Finally, this classification also underscores the necessity to confirm the absence of squamous markers, such as p40, in order to distinguish between LCNEC and squamous cell lung carcinoma, which is not a type of NET [65, 66].

Nevertheless, an emerging agreement over the last years in the NET field indicates that the current classification needs to be improved in order to incorporate NETs heterogeneity. One of the key aspects to be improved probably relates to the cyto-histology techniques currently used, many of which have been almost unchanged in the last decades. Several procedures should be applied from now on, such as fine-needle aspiration cytology (FNAC) or the incorporation of additional markers to classic Ki-67 (e.g. ATRX or Rb), which leads the way to the definition of a refined panel of markers and personalized therapies [67].

Indeed, despite providing further improvements and refinements to facilitate a better understanding and management of NETs, the current classifications are not free of controversy and discrepancies, as it can be seen, for example, in the recent review from the Spanish Tumor Registry (R-GETNE), where the impact of heterogeneity in the WHO classification is

highlighted [68]. Along these lines, it seems clear that current histopathological classification adds another important layer to NET heterogeneity by portraying not only the ample variety of tumor histotypes within a given class of NETs, but also, by exposing the differences that arise in the classifications among the different NET types (Fig. 1).

2.3 Clinical features

The clinical features associated with different types of NETs are also highly diverse. Although many low-grade lung NETs patients have no symptoms or only present non-specific respiratory symptoms at the time of diagnosis [7], occasionally, these patients may show cough, dyspnea, hemoptysis or wheezing, and functional tumors (lower than 10%) can exhibit hormonal symptoms, such as hyperhidrosis, flushing or diarrhea [7, 69, 70]. However, approximately 30% of low-grade lung NETs present metastasis at diagnosis. On the other hand, high-grade lung NETs are strongly associated with smoking, and although disease is normally advanced at diagnosis, and over 60% of patients show metastasis, symptoms are often similar to low-grade NETs [10]. In the case of GEP-NETs, a high number of patients, especially those with non-functioning tumors, remain without symptoms for years [71]. In contrast, patients with functioning tumors present symptoms related to the specific hormone secreted by the tumor and, usually, manifest a syndrome, such as hypoglycemia in insulinomas, peptic ulcer in gastrinomas, hypokalemia in VIPomas or diabetes in glucagonomas. When the tumor is sufficiently large, it may cause symptoms due to the tumor mass, such as pain, weight loss, nausea or bleeding [72, 73]. This patent clinical heterogeneity is closely interrelated to, and probably derives from, specific tumor features that, like location or cellular and molecular composition, are also correspondingly heterogeneous, which reinforces the notion that clinical NET complexity likely reflects the interaction of multiple layers of heterogeneity (Fig. 1).

2.4 Functionality

In close association with the clinical heterogeneity present among NET patients (Fig. 1), these tumors can be classified as functional or non-functional attending to their capacity to secrete hormones and/or amines and be consequently associated with secondary syndromes due to hormone hypersecretion [15, 74, 75]. Obviously, the specific hormone and/or amine secreted depends on the original location of the NET, although cases of ectopic secretion also exist. In addition, and irrespective of their tissue of origin, all NETs secrete some neuroendocrine-associated peptides (even in the case of non-functional NETs), such as CgA, neuron-specific enolase, alpha subunits and pancreatic polypeptide, which, despite their lack of functional and clinical effects, may serve as diagnostic markers [76]. In the past, functional NETs and hormonal syndromes were mainly attributed to pancreatic and ileal NETs, but it has been shown that these substances could originate from other tissues, increasing the complexity for diagnosis [77].

The main substance secreted by all types of NETs (but more prominently from midgut NETs) is serotonin, which is produced by tryptophan metabolism and has been found to be clearly oversecreted in some NETs [78]. This hypersecretion of serotonin is directly involved in the development of carcinoid syndrome, which is characterized by diarrhea and flushing, or even with carcinoid crisis or Hedinger's syndrome, two more severe pathologies [79, 80]. Other peptides that may be secreted by NETs are peptide substance P, kallikrein, tachykinins, prostaglandins, neurotensins, etc. [81, 82]. Additionally, histamine hypersecretion can also lead to atypical carcinoid syndrome [83]. Serotonin and histamine are commonly hypersecreted by gastrointestinal and lung NETs, leading to carcinoid syndrome, but other types of NETs can also present this hypersecretion, although in a limited proportion of cases [7, 14, 76].

Among GEP-NETs, pancreatic and duodenal NETs are especially relevant in terms of functionality, as a high proportion of NETs with these origins lead to syndromic outcomes. In particular, both organs may develop somatostatinomas, being more important in duodenum, and gastrinomas, which may lead to Zollinger-Ellison syndrome, because of the ectopic hypersecretion of gastrin. In addition, some particular functional NETs may appear in the pancreas, such as insulinomas, glucagonomas and VIPomas [74, 76, 77].

Finally, NETs can also course with ectopic hypersecretion of some hormone as is the case of paraneoplastic syndromes, which may be produced by secretion of ACTH, parathyroid hormone-related peptide or GHRH, producing Cushing's syndrome, hypercalcemia and acromegaly, respectively [76, 77].

2.5 Cellular and signaling heterogeneity

From a cellular point of view, it should be noted that, even from its normal physiological background, cells of the neuroendocrine system already display a remarkable heterogeneity (Fig. 1). Indeed, there are at least 17 known neuroendocrine cell types in the GEP tract. Some of them are only present in a particular organ, as is the case of A (α), B (β) and PP cells in pancreas; ECL and X cells in stomach; and I, M, N and S cells in the small bowel. On the contrary, other cell types may appear in several GEP organs, like G cells in stomach and small bowel; P/D1 cells in stomach, pancreas and small bowel; and Gr, GIP and L cells in small and large bowels. Furthermore, some of these cell types may be present in all GEP organs, as is the case of D (δ), EC and VIP cells [20]. Therefore, it is not only important to consider the organ of origin but also the cell type that particularly transforms to generate the NETs, in that tumors arisen in a specific organ

can be originated from totally distinct cells; while tumors located at different organs could be arisen from similar cell types, which adds an additional layer of heterogeneity to the complexity of NETs.

Interestingly, despite the remarkable heterogeneity of NETs, a molecular pathway has been found to be prominently altered in a vast majority of NETs, namely the mammalian target of rapamycin (mTOR) [84]. Specifically, the mTOR is a kinase dependent signaling cascade pathway involved in cell growth, comprised by two multiprotein complexes, mTORC1 and mTORC2 [85]. In this sense, mutations in key suppressors of this pathway (e.g. NF1, TSC2 or PTEN) [84, 86, 87] and altered expression of mTOR pathway components [84] are common hallmarks of a great proportion of NETs, wherein these alterations seem to be directly related with tumor development and progression.

However, NETs are also heterogeneous from the molecular point of view, as many other crucial pathways have been found to be altered in particular tumor types, which can increase their complexity and hamper their study and classification, as is the case of Notch pathway [88], whose interest is growing over the last years. Another classic example are the lung NETs in that NSCLC are usually related to mutations in KRAS [89], HRAS [90] and NRAS [91], while SCLC are more associated to RB1 mutations [92, 93].

2.6 Genetic

NETs are among the neoplasms with a more marked heritable component, having been associated with a minimum of ten different genetic syndromes [94]. One of the first syndromes described, most representative of these diseases, is Multiple Endocrine Neoplasia type 1 or MEN1 syndrome, an autosomal dominantly inherited complex endocrine syndrome. MEN1 syndrome is mostly associated to the appearance of pancreatic and duodenal NETs (40–80%), but mutations in MEN1 can also lead, also to a lesser extent, to lung, thymic and gastric NETs. Normally, tumor development is associated to the mutation of both MEN1 alleles, but thymic and duodenal NETs could appear without the complete inactivation of this gene [94, 95].

MEN2 syndrome is also an autosomal dominantly inherited disorder, which is associated to the development of multiple endocrine MTC, pheochromocytomas and parathyroid adenomas. MEN2 syndrome includes two subtypes, being type B more aggressive. MEN2 syndrome appears by the mutation of RET (a.k.a. Rearranging During Transfection), a protooncogene that encodes a tyrosine kinase receptor, which gains an autonomous activation, transducing its signal through RAS/MAPK and PI3K/AKT pathways. This mutation may occur in two different zones of the gene, which lead to the different syndrome subtypes. In addition, the Familial MTC (FMTC) syndrome is only associated to MTC, being less aggressive than MEN2, but it is also caused by a RET mutation [35, 94, 96–98].

Furthermore, there are many other NET syndromes, but they are much less common. Neurofibromatosis type 1 (NF1) syndrome is associated with several NETs types, such us duodenal NETs or pheochromocytomas [94] and it is linked to RAS and ERK/MAPK pathways. Another example is the Von Hippel-Lindau (VHL) syndrome, which is associated with pheochromocytomas, paragangliomas, pancreatic and other NETs [94] and is caused by the loss of VHL tumor repressor gene, which is related with HIF and VEGF pathways.

Thus, the diversity of genetically inherited familial syndromes adds another layer of inherent heterogeneity to NETs, which is intertwined with tumor location, as well as with functional, cellular and signaling NET feature (Fig. 1).

3 Approaches to understand NETs heterogeneity

The multilayered heterogeneity of NETs hinders their study, classification, and clinical management. Accordingly, great efforts are being directed towards obtaining a more complete picture of NETs, by deploying systematic studies, mostly based on *omics* approaches, searching for novel sources of information, more specific diagnosis and prognosis markers, and also aiming at establishing new histopathology consensus. These efforts are serving as well to unveil the existence of previously unknown factors that can contribute to NET heterogeneity, such as those associated to altered modulation of epigenetics or splicing processes.

3.1 Histology markers

Given the complexity of NETs, a combination of different markers is commonly necessary to appropriately define the type of tumor and to obtain relevant clinical information. Currently, different research groups are searching for novel, improved markers to complement the available, well-established ones, in order to better define and classify NETs [99].

The best-established histologic marker for NETs is Ki-67 (and its relation with mitotic count), which is useful to determine the grade and mitosis status of the tumor [3, 26, 31, 65, 100, 101]. Particularly, Ki-67 is commonly used and accepted as a marker in GEP-NETs [102]. However, despite being widely used, the Ki-67 index cut-off used to grade the tumor is different for every type of tumor, and even reference institutions (i.e. WHO and ENETS) do not reach a consensus in terms of grading of the same tumor type, which consequently leads to the appearance of different classifications [20, 26, 29]. Nonetheless, considerable efforts are being implemented during the last years to unify the different classifications of NETs. In addition, the role of Ki-67 as prognostic marker is still

uncertain, and current investigations are being implemented to clarify if this index can inform about the progression of the tumor [102].

There are several additional useful histologic markers for GEP-NETs, which may be also determined in plasma and are, therefore, used in the diagnosis and prognosis of NETs [103–105]. However, these markers also exhibit severe limitations, including the fact that none of them is ubiquitously expressed in all NETs. Particularly, the most used markers of these types of tumors are CgA, neuron-specific enolase (NSE), synaptophysin and serotonin. CgA is a protein present in the secretory granules of neuroendocrine cells. However, although it is the most used non-invasive biomarker for NETs, CgA is not over-expressed in all tumors, as is the case of distal colon or rectus, and, therefore, can generate false negatives. Besides, CgA may exhibit a reduced expression in tumors with low density of secretory granules and tumors with poor differentiation, which hampers its utility as general biomarker [103]. Synaptophysin, a protein related to small vesicles, is also a widely used non-invasive biomarker for NETs. However, it is expressed in normal and tumoral cells, which diminishes its specificity as NET biomarker, and, even worse, it is not very sensitive in the detection of stomach and duodenal NETs [103]. Similarly, NSE also exhibits reduced specificity as it is expressed in normal neuronal and neuroendocrine tissue, and it presents cross-reactivity with an isoform present in non-endocrine cells. In addition, its distribution in hindgut NETs is less abundant than in other NETs [103]. Finally, an additional widely used marker for NETs is serotonin and, particularly, its metabolite 5-hydroxyindole acetic acid (5-HIAA), which is excreted by urine, allowing measures with non-invasive tests. Nevertheless, its use is limited to serotonin secreting tumors and its levels are quite variable in different types of tumors [102, 103].

In the case of lung and thymus NETs, Ki-67 index is also used to classify the tumors by grade together with mitotic index, but the overlapping of Ki-67 expression in typical and atypical carcinoids hinders the distinction between well-differentiated lung NETs [7]. CgA is, again, an important biomarker for these NETs, as CgA usually exhibits a high increase in functional and non-functional tumors, reaching a 100% of specificity in some cases. However, CgA levels may be altered by some treatments (e.g. somatostatin analogs or proton pump inhibitors) [12]. NSE is also useful for lung and thymus NETs, although its release to serum seems to be unconnected to the secretory activity of the tumor [12]. Finally, serotonin or, more specifically, 5-HIAA is less useful than in other NETs, because it seems to be more or less constant in patients with lung or thymus NETs [12].

Importantly, although functional NETs express and secrete specific hormones and/or peptides that could be used as markers both in the tumor and in blood, it should be noted that normal cells can also express and secrete these hormones and/or peptides and, therefore, these markers do not present high sensitivity and specificity and, in many cases, they cannot properly define the functional status, so that additional clinical manifestations are required to finally determine and characterize tumor features [103].

3.2 Analysis of genetic factors

Recent studies have characterized the genetic factors that contribute to NETs tumorigenesis, revealing that a relatively limited number of mutations is normally presented in these tumors (reviewed in [94, 106]). Indeed, less than 30 mutations, approximately, have been identified so far as key mutations in NETs tumorigenesis [94, 106]. Interestingly, these genetic alterations seem to be tumor type-specific, thereby supporting the contention that these genetic factors could represent one of the basic mechanisms contributing to NETs heterogeneity. Of note, the mutations described seem to appear at different stages of tumor development, and are mainly associated to chromatin modifications, cell growth and tumor metabolism.

In the case of GEP-NETs, mutations in ATRX, DAXX, MEN1 or TP53 have been shown to be related to the development of progression of these tumors. Particularly, mutations in ATRX and DAXX, two genes related with alternative lengthening of telomeres, have been found in NETs, especially in PanNETs [107–109], wherein they seem to appear as late mutations in natural history of the tumor [94]. MEN1 is also commonly found to be mutated in NETs [110]; even though this mutation was discovered over 20 years ago, the function of its encoded protein, known as menin, still remain somewhat uncertain, although recent studies provide convincing data that relate it with transcription regulation [111]. Additionally, TP53 mutations are also present in these pathologies and, although they seem to be less relevant than in other tumors, TP53 mutations increase in poorly differentiated tumors [15, 94, 112]. In contrast, small bowel NETs markedly differ from the rest of GEP-NETs in this context, as the mutations described previously do not seem to play an important role in these tumors, while mutations in CDKN1B gene, which controls cell cycle, seem to be more relevant [94, 113], which further supports the high heterogeneity displayed by NETs.

In the case of pheochromocytomas and paragangliomas, the most relevant mutations have been found in genes related to metabolism, such as those found in succinate dehydrogenase subunits (SDHA, SDHB, SDHC, SDHD) and fumarate hydratase (FH) [94, 114]. ATRX has also been found mutated in a proportion of these tumors [110, 115]. However, even in these highly related tumors, important differences in carrying mutations have been described as is the case of HIF2α, a gene related to hypoxia found to be mutated in pheochromocytomas [114].

3.3 Analysis of epigenetic modifications

Epigenetics represents a regulatory mechanism that controls gene expression without altering permanently nucleic acid sequence [116]. This regulation is mediated by selective and reversible modifications of DNA sequences and proteins (mainly histones), which control the conformational transition between transcriptionally active and inactive states of the chromatin. In the particular case of NETs, this regulatory mechanism has gained considerable attention, mainly due to the low frequency of classical mutations, such as TP53 or KRAS. In this scenario, as expected, the diversity in epigenetic modifications altering different types of NETs is remarkably high, in that particular NET subtypes are characterized by different epigenetic patterns and some NET subtypes do not even present a recognizable pattern [117]. Thus, epigenetic regulation comprises a novel layer of heterogeneity for NETs, which adds further complexity to the nature and the precise understanding of these tumors but also provides a novel source of information to better classify NETs, and a potential actionable target for their future treatment.

In PanNETs, hypermethylations (one of the main epigenetic modifications found in DNA) have been reported in the promoter of Ras-association domain gene family 1 (RASSF1), a tumor suppressor gene frequently found to be silenced by epigenetic mechanisms in several cancers. This hypermethylation has been reported to be higher in non-functioning and metastatic tumors, therefore suggesting its clinical relevance [117–120]. Another example is the epigenetic modification of cyclin-dependent kinase inhibitor 2A (CDKN2A), a regulator of the cell cycle and tumor suppressor gene, wherein the methylation of its promoter is frequently found in pancreatic NETs [117, 120]. In this regard, it is worth noting that insulinomas seem to exhibit a particular epigenetic pattern, that is not shared with other PanNETs, is associated with hypermethylation of IGF2, and could be related to the development of these tumors [117, 121].

On the other hand, gastrointestinal NETs display a very different pattern of methylation. A pioneering study by Chan et al. (2003) suggested that several tumor-related genes were differentially methylated in gastrointestinal compared to pancreatic NETs, including MGMT, THSB1 or CDKN2A genes [117, 122]. A detailed selection of different methylations in pancreatic and gastrointestinal NETs is summarized in [123]. Of particular relevance are the methylations in cadherin-associated protein CTNNB1 promoter as they are frequent in metastatic tumors. Interestingly, hypermethylation of the promoter of other cadherin-associated protein, CDKN2B, have been found to be related with low grade pulmonary NETs [117]. Therefore, although these NETs could share some specific epigenetic modifications, like those found in RASSF1 promoter, which are also found in gastrointestinal [123] and pulmonary [117, 124] NETs, and could represent an epigenetic hallmark of foregut NETs, recent reports suggests that the heterogeneity in these epigenetic patters in NETs could be used as marker to categorize different types of tumors [125].

3.4 Analysis of post-transcriptional regulation mechanisms

The post-transcriptional regulation of gene expression exerted by microRNAs (miRNAs) and long non-coding RNAs (lncRNA) is an additional mechanism potentially involved in NET heterogeneity. However, the presence and role of these regulatory molecules have not been explored in NETs as profoundly as in other tumoral pathologies. miRNAs are short non-coding RNAs that regulate gene expression post-transcriptionally. Several reports have shown that miRNAs are differently dysregulated in NETs subtypes [126, 127] and, even, that the same miRNA can be dissimilarly altered in different tumors, demonstrating the intrinsic heterogeneity of miRNAs expression in NETs [117]. As a case in point, miR-103 and miR-107 are exclusively overexpressed in pancreatic NETs, whereas miR-204 is only overexpressed in insulinomas [128]. In addition, other miRNAs show opposite regulation in different NETs, e.g. miR-155 is downregulated in metastatic ileal NETs but upregulated in high-grade lung NETs [117, 128–130]. In this context, it has been proposed that the profile of different miRNAs could represent putative biomarkers for NETs, but the high heterogeneity suggests that these profiles could be completely different among particular NETs subtypes [131, 132].

LncRNAs are non-coding RNAs with more than 200 bp of length that also regulate gene expression post-transcriptionally. Knowledge regarding lncRNAs and their function is increasing exponentially nowadays; however, few studies have addressed this subject in NETs. A recent study demonstrated a reduction of the lncRNA MEG3 in PanNETs, wherein the epigenetic activation of lncRNA MEG3 could represent a therapeutic option for treating PanNETs and insulinomas [133]. Nevertheless, implication of lncRNAs in NETs tumorigenesis and their potential utility as diagnostic/prognostic biomarkers or therapeutic targets are still to be further explored.

3.5 Analysis of alternative splicing processes

Splicing is a co-transcriptional process through which the precursor mRNA is converted into mature mRNA by eliminating the introns and assembling the exons. This process is catalyzed by a macromolecular complex, named spliceosome, that is composed by several small nuclear RNAs (snRNAs) and associated proteins [134]. Additionally, this process is regulated by splicing factors, which modulate and complete the action of the spliceosome [135]. The process of canonical splicing is commonly expanded to generate, from the same precursor, several different mature mRNA (i.e. alternative splicing variants) with similar, different or even opposite

functions. This process, termed *alternative splicing*, drastically increases the complexity and versatility of genome, and can also provide an additional level of heterogeneity for NETs [136, 137]. Moreover, the incorrect functioning of this machinery may lead to altered alternative splicing, which can result in the production of aberrant protein variants, some of which have been found to be associated to different diseases, including cancer [138–140]. Indeed, recent studies have revealed the presence in NETs of different aberrant splicing variants related to tumor development and aggressiveness, which may be linked to alterations in the splicing machinery. However, there are still scarce reports and, therefore, more studies are needed in this field. In particular, it has been shown that aberrant variants of ghrelin [141] and somatostatin receptor subtype 5 [142] are overexpressed in pancreatic NETs, wherein they are associated with tumoral and aggressive features; indeed, functional experiments demonstrated their capacity of enhance malignant characteristics, such as cell proliferation, migration or serotonin secretion. In addition, it has been published that some variants are only expressed in one particular type of NETs, such as a variant of CD44, named CD44v6, that is exclusively altered in gastrinomas [143, 144]. Also, in lung NETs, it has been suggested that a splicing variant of ACTN4 could be used as diagnostic and prognostic tool in these tumors [145]. Additionally, as mentioned above, specific splicing factors have been also found to be dysregulated in NETs, as is the case of the serine/arginine rich splicing factor 2 (SRSF2), which cooperates with the transcription factor E2F1 to modulate cell cycle progression and control apoptosis in NSCLCs [146]. In this regard, although the actual impact and relevance of the presence of splicing variants and dysregulations in splicing machinery are still to be defined, it seems reasonable to expect that these alterations will likely add a further layer of heterogeneity in NETs.

4 NETs heterogeneity as an opportunity

As reviewed above, current evidence indicates that NETs comprise an ample group of tumors that are highly heterogeneous from different points of view: location, histopathology, cellular and molecular features and clinical implications (Fig. 1). This heterogeneity greatly hinders the efforts implemented to characterize, classify and study NETs from a basic, translational and clinical perspective. In addition, their reduced incidence compared with other tumoral pathologies further hampers their analysis and the establishment of more solid and informative clinical trials, which are also influenced by this heterogeneity [147]. However, NETs heterogeneity may also be viewed as an opportunity to improve and implement personalized precision medicine, since each patient could benefit from a more tailored treatment if the tumor is comprehensively characterized considering all the mentioned levels of heterogeneity.

Specifically, the marked heterogeneity of NETs hinders the proper diagnosis and the appropriate prediction of the prognosis of NET patients, as there are no universal biomarkers that could precisely determine tumor presence and anticipate progression. For this reason, new approaches and further studies are urgently required in order to identify and validate new biomarkers and to improve the available tools. In this regard, *omics* have gained great importance over the last years, due to the significant amount of valuable information that they can provide [112, 148]. Indeed, with all this novel information available, new panels of biomarkers are emerging, through the identification of combination of molecules, mutations, and/or changes in expression levels that may help to better classify NETs, anticipate tumor appearance, or predict their development, progression and/or response to treatment. This is the case of NETest, an assay developed through a series of multidisciplinary studies that propose the measurement of circulating transcripts to predict response of the patient to somatostatin analogs [149]. In addition, there are other examples in the literature using circulating miRNAs in small bowel NETs [150] or genomic alterations in metastatic gastroenteropancreatic NETs [151]. Therefore, these new approaches are suggested as the future avenues to identify the definitive tools to diagnose and classify NETs, to improve prediction of their prognosis and to select the more appropriate treatment.

The same rational applies to the case of NET treatments, which are profoundly influenced by the heterogeneity observed in these tumors, as is reflected in the bibliography [152, 153] but also in the guidelines for the management of NET patients, such as those from ENETS [3, 154] and from the National Comprehensive Cancer Network (NCCN) [155]. Indeed, the intrinsic heterogeneity characterizing NETs is pointing the need towards the definition of a more personalized medicine for the treatment of NET patients, as elegantly reviewed by Pavel and Sers [156]. Specifically, surgery is the first-line treatment in NETs, especially in functional NETs, but it is also considered in non-functional tumors in order to alleviate symptoms [155, 157]. On the other hand, although chemotherapy is not widely used for the treatment of NETs, being 5-Fluorouracil and streptozotocin the most frequent agents used [157], other medical treatments are widely used in NET patients. This is the case of somatostatin analogs (SSAs), and more specifically octreotide and lanreotide, which have been shown to decrease tumor proliferation when somatostatin receptors are expressed in the tumor [158]. More recently, peptide receptor radionuclide therapy (PRRT), which is a novel approach based on a combination of a SSA and a beta radiation emitting radionuclide, has been shown as a more specific and efficient approach than SSAs [159–162]. Nevertheless, and further supporting the intrinsic heterogeneity of NETs, somatostatin receptors are not always present or in the same

proportions in NETs and, therefore, efficiency and applicability of these SSAs-based therapies are considerably reduced [163–165]. The same scenario appears in the case of alternative therapies targeting specific pathways found to be altered in these tumors, such as everolimus and sunitinib [166, 167]. Everolimus is a specific inhibitor of mTOR, the main protein of one of the most altered pathways in these diseases [168], while sunitinib is a multiple tyrosine kinase inhibitor [169]. Both therapies have shown promising effectiveness in certain proportion of NET patients; however, these drugs are still insufficient to overcome the levels of NET heterogeneity (as many patients do not benefit from these therapies) and further investigation deems necessary [156]. For this reason, novel and future therapeutic avenues will have to encompass the combined and/or sequential use of multiple targets, wherein the characterization of the intrinsic heterogeneity of each NET seems to be crucial in order to develop a more precise and tailored personalized medicine.

5 Conclusions

In this review, we tried to summarize the most significant lines of evidence supporting the remarkable and intrinsic heterogeneity of NETs (Fig. 1), which affects all the relevant aspects of these tumors and has important implications in the development, progression and medical treatment of the diseases. The knowledge gathered to date leads to conclude, firstly, that heterogeneity is an intrinsic and recognizable hallmark of NETs. Secondly, it is apparent that the origin of NET heterogeneity is multiple and derives from several interconnected levels, ranging from locational to clinical, from genetic to epigenetic and from functional to morphological. The complex crosstalk among the different layers of heterogeneity of NETs hampers their study and hinders the identification of more sensitive and specific biomarkers and therapeutic targets. Accordingly, heterogeneity must be considered as a key factor to understand tumor biology and for the future approach and development of therapeutic strategies. Clearly, NETs can no longer be viewed as a single disease entity, and their diagnosis, prognosis and treatment must reflect this multilayered heterogeneity.

Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare in this work.

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