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CICLO XXXII

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Pancreatic and broncho-pulmonary neuroendocrine neoplasms in vitro studies:

a route from culture systems evaluation

to approved and innovative therapeutic approaches

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1. INTRODUCTION

1.1 NEUROENDOCRINE NEOPLASMS

Neuroendocrine Neoplasms (NENs) are neoplasms arising from cells of the diffuse endocrine system (DNES) and represent a wide group of malignancies originating from different parts of the human body [1,2]. The concept of neuroendocrine system has been developed during the last 130 years starting from the word “nevrism” introduced first by Pavlov (1849-1936) in 1883 [3]. In those years the issue that was challenging scientific community concerned the link between the nervous system and body functions regulation. Several members of scientific community gave a contribution in the identification of those agents [4]. The existence of small granular yellow staining cells throughout the gastrointestinal (GI) tract was reported by Adolphe Nikolas (1861–1939) and Nikolai Kulchitsky (1856–1925) starting to develop the idea of a diffuse endocrine system [5, 6]. The discovery of Enterochromaffin and Argentaffin cells as well as of malignancies derived from them strengthened the idea of a neuroendocrine cell system spread throughout the body [4]. A very important step toward the identification of the neuroendocrine cells was made when Anthony Pearse (1916-2003) developed the amine precursor uptake and decarboxylation (APUD) concept. In particular, Pearse recognized that the different endocrine cells of the gut were linked together by a cytochemical capability: uptake the 5-hydroxytrytophan (5-HTP) and promote its decarboxylation to 5-HT [7]. APUD cells were postulated as being of neuroectodrmal origin and were named neuroendocrine cells [8]. However, even if it was proved that the diffuse neuroendocrine system is not derived from the neural crest, it is undeniable that neuroendocrine cells show typical characteristics of both endocrine and nervous system [9]. Indeed, neuroedocrine cells are currently described as

- cells able to produce neurotransmitters, neuromodulators and neuropeptides
- cells with dense core rich in granules from which the hormone is produced and secreted by

exocytosis in response to external stimuli

- cells with absence of axons and synapses [10]

Meanwhile the gastroenteropancreatic (GEP) system was described, Oberndorfer (1876-1944), a German pathologist, reported a series of uncommon tumours [11]. The scientist observed a difference between cancerous tumours of the ileocolonic junctions (carcinomas) and a group of ileum malignancies characterized by a less aggressive profile (carcinoids). These lesions were named as "karzinoide" (carcinoma-like) and described as benign tumours unable to metastasize [12]. The author subsequently revised this observation and, at the same time, several scientists all over the world started to report the existence of tumours with similar characteristics [12, 13]. Theodor Langhans (1839-1915), Otto Lubarsch (1860-1933) and William B Ransom (1860-1909) haves all observed lesions of the small intestine starting to develop the concept of malignancies derived from neuroendocrine cells (APUD cells) [4, 14].

1.1.2 CLASSIFICATION OF NENs

Classification of NENs has been, since their discovery, very challenging for the scientific community. NENs heterogeneity represented the most problematic feature in the elaboration of a global accepted classification and, during the past 5 decades, several approaches have been proposed [10]. Carcinoid tumours have traditionally been classified based upon their embryotic origin, morphologic pattern and silver staining affinity [14]. E. D. Williams and Merton Salander were the first that proposed in 1963 a classification of NENs based on their embryotic origin; according to this parameter, NENs were classified in three groups: tumours of the foregut (respiratory tract, thymus, stomach), tumours of the midgut (small intestine, appendix, proximal colon) and tumours of the hindgut (distal colon, rectum, genitourinary tract) [15]. Unfortunately, analysing morphological, functional and clinical characteristics of NENs, this classification

was soon considered inappropriate and restricted. At that time the generation of a standard classification system was essential for the correct management of those “new” malignancies.

World Health Organization (WHO) in 1980 made the effort to clarify NENs classification applying the term carcinoid to most of NENs with the exception for the endocrine tumour of the pancreas and thyroid, paragangliomas, small-cell lung carcinomas and Merkel cell tumours of the skin. Carcinoids were divided in groups: enterocromaffin (EC cells), gastrin (G cells) and other not specified carcinoids [14]. However, this classification only managed to introduce more terminological confusion since pathologist applied the word “carcinoid” to all tumours of the endocrine system while clinicians in general conflated it with the presence of carcinoid syndrome (constellation of signs and symptoms determined by a neuroendocrine neoplasms) [16]. Moreover, the growing scientific research in this area, was confirming the wide NENs heterogeneity leading to the consciousness that was almost impossible to equate NENs with a different origin site. In 1999, the Travis-WHO classification divided thymic and pulmonary NENs into four subtypes characterized by several degrees of aggressiveness: typical carcinoid tumours with a low grade of aggressiveness, atypical carcinoid tumour with an intermediate grade of aggressiveness and a more aggressive clinical behaviour, large cell neuroendocrine carcinoma (LCNEC) and small cell neuroendocrine carcinoma (SCLC) with high aggressiveness and poor prognosis [10]. A revised GEP classification was made by WHO in 2000. In this case the word “carcinoid” was avoided in favour of “NET” (neuroendocrine tumour) and malignancies were classified into three histologic categories regardless the site of origin: well differentiated neuroendocrine tumour with probably benign behaviour, well differentiated neuroendocrine tumour with uncertain behaviour and poorly differentiated neuroendocrine carcinoma with high grade malignant behaviour [17]. In this classification hormonal activity was taken in account but not proliferation index (Ki-67) that was however considered a strong prognostic factor. Therefore, in 2004, the WHO divided neoplasms of the lung in three grade based on mitotic index and necrosis and in 2010 another classification was

revealed with the contribution of the European Neuroendocrine Tumour Society (ENETS) [18, 19]. In this last version all tumours were considered malignant with the potential to metastasize and tumour grade was based on proliferation (i.e. the Ki-67) and mitotic count [18]. In this new classification system, the terms neuroendocrine tumour and neuroendocrine carcinoma (NEC) replaced the terms well and poorly differentiated tumours [20]. The classification included a staging system: well-differentiated tumours were instead called G1 or G2 NET and the most malignant tumours, which in WHO 2000 were classified as poorly differentiated NECs, were called large cell or small cell type G3 NECs [21, 22, 23]. Two other categories include mixed adenoneuroendocrine carcinomas (MANECs) and hyperplastic and preneoplastic lesions [22, 24]. 2010 classification represented a deep innovation since several key parameters were introduced to classify malignancies in different tumour grades [25]. Ki-67 antigen is a 359-kD non-histone nuclear protein with short half-life that plays an essential role in cell proliferation control and timing. This protein undergoes under a complex mechanism of post-translational phosphorylation and dephosphorylation by cell cycle proteins leading to its subcellular redistribution during mitosis and meiosis. Since its expression has been noted in all phases of cellular mitosis and not in quiescent cells (G0), it is used as a surrogate marker of proliferation. Ki-67 proliferation index refers to the percentage of cells that are positive by IHC for this antigen in a tumour section [26]. Using this parameter three tumour categories were identified: G1: Ki-67 index ≤2%, G2: 2- Ki-67 index between 3 and 20% and G3: Ki-67 > 20% [27, 25]. Tumour grading identification was also improved thanks to the evaluation of the mitotic rate defined as number of mitoses per ten high-power microscopic fields (HPF). Mitotic rate values for the three tumour categories were: G1: <2 mitoses per 2 mm^2 , G2: 2-20 mitoses per 2 mm^2 and G3: ≥21 mitoses per 2 mm^2 [28]. Evaluation of Ki-67 and mitotic rate were the most important criteria on which ENETS elaborated the staging system that was also enriched by the assumption that G1 and G2 tumours should have an intense expression of the two general immunohistochemical neuroendocrine markers: chromogranin A (CgA) and sinaptophysin. G3 carcinomas were instead

associated with necrosis, reduced expression of CgA and high expression of sinaptophysin [25]. However, since the assessment of malignancy differentiation degree was challenging, several biomarkers were reported as useful in understanding patients prognosis. p53 is a well-known tumour suppressor gene that encodes for its related protein that, in the wild type form, is really useful cell cycle progression control and genes stability maintenance [29]. However, the mutated form of p53 has been reported to be associated to a poor prognosis and higher degree of metastases in several tumour types including NEC: in high grade pulmonary NECs 70–100% of tumour cells have been shown to be mutated [30]. Since the mutated protein is not degraded, it accumulates into tumour cell nuclei resulting in an immunohistochemically detectable expression of the p53 protein. Therefore, p53 positivity at immunohistochemistry has been evaluated, with Ki-67, as a negative prognostic factor associated with a shorter Progression Free Survival (PFS) and Overall Survival (OS) [31].

The new WHO classification gave an important contribution towards defining the different tumour biology of NENs but further efforts were necessary to improve the prognostic assessment of each individual NEN. No formal tumour-nodes-metastasis (TNM) based staging systems was in use for GEP neoplasms until the American Joint Committee on Cancer (AJCC) formally adopted one in 2010 for all anatomic sites [32, 33]. This system matched the one recommended by European ENETS for GEP-NENs in 2006 [34]. However, the AJCC classification did not apply to high grade (large cell and small cell) neuroendocrine carcinomas and didn't exactly follow the ENETS classifications for some of the anatomic sites [35]. Different staging parameters generated a deep discrepancy and were not justified from clinical data [32]. The existence of two systems, each of which used identical TNM terminology, resulted in misunderstandings among clinicians generating the need of a common TNM staging system [36]. Between the two classifications, the one given by ENETS for GEP-NENs in 2006 was validated by several studies, and its biological relevance and power to discriminate different prognostic groups was largely confirmed [36, 37]. TNM classifications were

always considered essential in clinical practice since are used in order to assemble results of diagnostic procedures in a reproducible manner. This system represented the key element to connect diagnostic results, obtained by pathologists, and the clinical course of action. Therefore, the elaboration of a unique system in the future is desirable and necessary (38).

The acronym TNM for NENs staging proposal, according to ENETS guidelines described by Rindi et al. in 2006 (27), was referred to:

- *T - Primary tumour:* indicates the size of the primary tumour and the local invasion described as degree of diffusion into nearby tissues. An X after the T (TX) generally means that the primary tumour cannot be assessed while T0 indicates that evidences of primary tumour were found. This indicates the presence of a carcinoma in situ. T1, T2, T3, T4 describe the extent and/or size of the main tumour (39, 40).
- *N - Regional Lymph Nodes:* indicates the absence or presence and extent of regional lymph node metastases. NX means that regional lymph nodes cannot be assessed, N0 indicates that no regional lymph node metastases are present and N1–N3 specifies the increasing involvement of regional lymph nodes (40).
- *M - Distant Metastasis:* indicates the absence or presence of single or multiple metastases at any distant anatomical site (including non-regional nodes) (41).

In 2017 the WHO introduced a new classification for several tumours of endocrine organs including neoplasms of the endocrine pancreas. This classification is similar to the one presented in 2010 with three major updating (4, 42):

- Revision of Ki-67 cut-off value between G1 and G2 NET from 2% to 3 %
- Introduction of a new category NET G3 characterized by well-differentiated neoplasms with a Ki-67 > 20%
- Terminology revision regarding the mixed forms from MANEC to MiNEN (mixed neuroendocrine-non neuroendocrine neoplasm). MiNEN group includes neoplasms in

which the two components, neuroendocrine and non neuroendocrine, are both represented in at least the 30% of malignant cells

This new classification improved treatment and management of patients with pNEN and it is expected that the forthcoming WHO classification will apply the same grading system to other NENs [43, 44].

1.1.3 EPIDEMIOLOGY OF NEUROENDOCRINE NEOPLASMS

Although NENs have always been considered rare, based on the current medical literature, their worldwide incidence seems to have increased. Current incidence rates range from 3.24/100,000 in North Europe to 5.25/100,00 in USA [38]. In particular, in the SEER database, the annual age-adjusted incidence increased from 1.03/100,00 in 1973 to 6.98/100,00 in 2012. A similar significant increase over time has been reported from other authors in different geographic areas. For example, in United Kingdom, a growth in incidence from 3.9 per 100,000 in 2001 to 8.8 per 100,000 in 2015 has been demonstrated [45]. The incapability to collect complete data about NENs epidemiology is mostly due to their heterogeneous classifications in different countries and to the different methods of patient identification [38, 46]. In total, approximately 1% of all malignancies are NENs. Register studies in the last 30 years showed an annual increase in incidences of approximately 3–4% and this increase is due to several factors [47]. The increase in incidental diagnoses, a better clinical awareness, a widespread use of cross-sectional imaging/endoscopic techniques, an increased plasma biomarker measurement (CgA) and more accurate histopathological diagnosis have certainly contributed to a huge increase in their incidence [48]. NENs tumour heterogeneity and nonspecific symptoms presentation are the main reasons of the frequent delay in diagnosis up to 7 years. As a result, malignancies are often discovered in an advanced stage when a cure is no longer possible [49]. Gastrointestinal tract represents the primary site of NENs (61%) while the lung is the second most common site (23%); the remaining percentage is from other organ systems. The most common

primary NENs sites in the digestive tract are: small intestine (30.8%), rectum (26.3%), colon (17.6%), pancreas (12.1%) and appendix (5.7%). At diagnosis time, according to the SEER database, 53% of patients with NENs present with localized disease, 20% display locoregional disease and 27% have distant metastases. Patients with a family history of NENs in a first-degree relative have a 3.6-fold increased risk of disease (33, 50). Considering NENs heterogeneity, OS is different for each tumour. In general, patients with poorly differentiated tumours and distant metastases have showed a lower OS than those who were affected from well-differentiated and localized tumours. Unfortunately, the general OS for patients with metastatic disease at time of diagnosis is only 5 months (51). However, the survival rate has improved in the last two decades and population-based studies have showed that median OS for metastatic pancreatic and small bowel NENs is 24 and 56 months respectively (52). NENs are therefore a huge health problem despite having been always been described as rare.

1.1.4 RISK FACTORS AND NEUROENDOCRINE NEOPLASMS SYNDROME

Several risk factors have been recognized in NENs development. Family history of cancer appears to be the most relevant risk factor for NENs at all the investigated sites. Increased risk of NENs, especially for specific anatomical sites, has been documented to be associated with alcohol consumption and cigarettes smoking. In particular, alcohol intake represents a risk factor for rectum and pancreas NENs while cigarettes smoking have been identified as a risk factor for small intestine, pancreas and some types of bronchial NENs (38). NENs are sporadic in most of the patients, but sometimes are part of specific hereditary tumour syndromes such as multiple endocrine neoplasia type 1 (MEN1), multiple endocrine neoplasia type 2 (MEN2) and multiple endocrine neoplasia type 4 (MEN4). Moreover, pNENs seem to be related in a minority of patients with von Hippel-Lindau disease. Some NENs cases were also observed in Recklinghausen

neurofibromatosis (neurofibromatosis type I) and tuberous sclerosis. Other genetic syndromes such as Carney complex, non-MEN1 familial isolated hyperparathyroidism (FIHPT) and Conn adenoma have been postulated during the last years to be associated with NENs development but this hypothesis has never been confirmed.

- MEN1: MEN1 is a syndrome caused by germline mutations of the MEN1 gene that encodes for menin, a tumour suppressor protein with several functions not yet completely elucidated. It is well known that menin is expressed ubiquitously and is a regulator of gene transcription. The most accredited hypothesis concerning its function is that menin can act as a suppressor of telomerase expression. Telomerase is an enzyme that represents a very important factor in the maintenance of telomeres length during cell division and its inactivation could lead to cell immortalisation and tumour development [53, 54]. Menin mutations are the cause of MEN 1 syndrome that is an autosomal dominantly inherited syndrome characterized by the occurrence of tumours involving two or more endocrine glands within a single patient. The classic MEN1 spectrum includes tumours of parathyroid glands, anterior pituitary, endocrine pancreas and endocrine duodenum. Less frequently observed neoplasms include neuroendocrine tumours of lung, thymus and stomach as well as some non-endocrine tumours (lipomas, angiofibromas, ependymomas) [55]. Parathyroid tumours are the most common feature of MEN1 and occur in 95% of patients while pancreatic islet tumours represent the 40% of patients and anterior pituitary tumours the 30% [56].
- MEN2: MEN2 is defined by the occurrence of medullary thyroid carcinoma (MTC), pheochromocytoma, and hyperparathyroidism caused by parathyroid gland hyperplasia/adenoma (PHPT). MEN2 is further divided into MEN2A that typically manifests with MTC, pheochromocytoma, and PHPT, and MEN2B that manifests with MEN2A features, although typically lacking PHPT, ganglioneuromas of the lips, tongue and

colon [57]. MEN2 is an autosomal dominantly inherited cancer syndrome and the gene responsible of syndrome occurrence is RET. The gene localized at chromosome 10q11.2 and encodes for a receptor tyrosine kinase, RET protein, able to activate a complex downstream signalling pathway that includes RAS/extracellular signal-regulated kinase (ERK). This pathway promotes cell survival and proliferation. RET mutations lead to its overactivation and to the development of several malignancies [58].

- MEN4: MEN4 syndrome is caused from mutations in CDKN1B gene that encodes for the protein $p27^{kip1}$ commonly called p27 or KIP1. This protein has a tumour suppressor role and regulates cell cycle progression. A loss of p27 function cause a non-inhibition of the complexes Cyclin E-CdK2 and Cyclin D-CdK4 leading to a deregulate cell cycle and malignancy development [57]. Two of the most common phenotypic features of patients with MEN4 are parathyroid and pituitary neoplasia. Recently, mutations in CDKN1B were also identified in patients with sporadic PHPT, lymphoma, and breast cancer, demonstrating a novel role for CDKN1B as a tumour susceptibility gene neoplasms with endocrine origin and others [59].
- Von Hippel-Lindau disease (VHL): VHL disease is an autosomal dominant disorder that leads to the development of a variety of tumours and cysts in visceral organs and central nervous system. Brain, kidney, pancreas, adrenal gland, and epididymis are the main organs hit in VHL disorder. During their growth, these tumours impair the function of the primary organs and sometimes metastasize to distant organs, and thus are thought to have malignant potential. The gene for VHL syndrome was identified in 1993 and localized to chromosome 3p25.5. The gene codifies for pVHL that has an important function in HIF1α protein regulation. Mutations in pVHL lead to an over activation of HIF1α that is a transcription factor able to promote transcription of a high number of genes including Vascular Endothelial Growth Factor (VEGF), Platelet Derived Growth Factor (PDGF) and

erythropoietin. Since these genes, and related proteins, are all involved in glucose uptake metabolism, a dysfunction in pVHL leads to abnormal cell growth and metabolism [60, 61].

- Neurofibromatosis type 1 (NF1): NF1, or von Recklinghausen disease, is an autosomal dominant disorder that affects 1/3000 livebirths [62]. The protein associated with NF1, neurofibromin, is a tumour suppressor able to block RAS protein. Since RAS is a key regulator of the intracellular PI3K/AKT/mTOR pathway, NF1 plays a pivotal role in regulating cell cycle progress and apoptosis. Loss of function of the NF1 gene results in mTOR up-regulation, loss of cell cycle control and, therefore, tumour development [63]
- Tuberous Sclerosis Complex (TSC): TSC is an autosomal dominant genetic disorder, characterized by the development of hamartomas, benign tumours, and rarely, malignant tumours in multiple organs. The disorder is caused by a mutation in TSC1 and TSC2 tumour suppressor genes resulting in loss of the respective encoded proteins, hamartin and tuberin, and lacking of deactivation of the mammalian target of rapamycin complex 1 (mTORC1) pathway. Since mTORC1 promotes cell growth and survival, its missing deactivation results in abnormal cell growth and tumours development [64, 65].

1.1.5 CLINICAL PRESENTATION: CARCINOID SYNDROME

Another NENs classification is based on their capability to produce/secrete amines and peptides. According to these parameters NENs are divided in two categories: functioning or non-functioning neoplasms. Neoplasms not associated with a hormones overproduction or not associated with an obvious syndrome are called non-functional whereas neoplasms characterized by a hormones overproduction are referred as functional and may be associated with distinct clinical symptoms/syndromes [66]. Hormones hyper production is commonly one of the main factors that contribute to an earlier diagnosis in comparison with non-functioning counterparts [25]. Patients

with functional NENs may develop Carcinoid Syndrome (CS) that is a paraneoplastic disease associated with the secretion of several factors, such as polypeptides, vasoactive amines, and prostaglandins. The recent US epidemiological study reported an estimated CS prevalence of 19% in a population of 9512 patients with NENs diagnosis [67, 68]. The main symptom of CS is episodic facial flushing that may be accompanied by hypotension and tachycardia, diarrhoea, bronchoconstriction, venous telangiectasia, dyspnoea and fibrotic complications such as mesenteric/retroperitoneal fibroses and carcinoid heart disease (CHD) [68, 69]. The main CS mediator appears to be serotonin (5-HT), which is considered the primary marker associated with the syndrome, as well as histamine, prostaglandins, and tachykinins. However, over 40 substances were identified as being potentially involved in pathogenesis of CS [67]. There are unfortunately limited treatment options for patients with CS. For several decades, patients with NENs and CS have been first treated with somatostatin analogues but, despite these agents provide significant relief from CS symptoms, are not capable to stop tumour progression. Therefore, new therapeutic approaches are needed. Telotristat is a novel oral inhibitor of tryptophan hydroxylase, which is the rate-limiting enzyme in serotonin synthesis. Other approaches to control CS include the use of peptide radioreceptor therapy (PRRT) using radiolabeled somatostatin analogues, the use of MIBG (meta-iodobenzylguanidine) as well as other therapeutic options including liver-directed therapies (radioembolization, chemo-embolization/embolization) [70, 71].

1.2 GASTROENTEROPANCREATIC NEUROENDOCRINE NEOPLASMS

GEP-NENs are malignancies derived from neuroendocrine cells spread in the entire gastrointestinal tract that represent 1-4% of all gastrointestinal neoplasms [72, 38]. GEP-NENs comprise a heterogeneous family of neoplasms with a wide and complex spectrum of clinical behaviours and distinctive histopathological features in comparison with those displayed by conventional

gastroenteropancreatic epithelial cancers [73]. The small intestine (30.8%), rectum (26.3%), colon (17.6%), pancreas (12.1%), and appendix (5.7%) are the most common primary NENs sites in the digestive tract [74]. GEP-NENs represent the second most common digestive cancer in terms of prevalence and SEER program of the National Cancer Institute reported that annual age-adjusted incidence rate grew from 1.09 per 100,000 in 1973 to 6.98 per 100,000 in 2012 [33]. Also in this case the specific reason for the increase in diagnoses remains unclear but trends in imaging and improved recognition of neuroendocrine histology are likely to play a role. GEP-NENs may arise sporadically or as the result of hereditary predisposition syndromes such as MEN1, VHL disease or NF1 and their diagnosis is generally at a young age when compared with carcinomas [72]. No environmental risk factors have been definitively identified so far. As previously indicated, GEP-NENs are characterized by their ability to produce, store and secrete a large number of peptide hormones and biogenic amines which can lead to the development of distinct clinical syndromes [72].

1.2.1 CLASSIFICATION OF GASTROENTEROPANCREATIC NEUROENDOCRINE NEOPLASMS

GEP-NENs were classified in 2010 by WHO classification elaborated under the auspices of the ENETS proposals. The AJCC staging manual, seventh edition, rapidly followed. The 2010 WHO/AJCC classification introduced several principles such as the assumption that diagnosis process should be guided by the observations of tissue sample morphologic features and immunohistochemical analysis for specific biomarkers (mostly CgA and synaptoshysin) [38]. WHO/AJCC established that the grade of NENs differentiation was mainly defined on the basis of two factors: Ki-67 proliferation index (calculated in the areas with the highest number of labelled cells) and the mitotic activity. How to make Ki-67 and mitotic index count was widely debated

and, in order to avoid inaccuracy, tumour grade has been recommended to be measured in the most mitotically active areas of the pathology specimen [33]. Grading classification was the follow [75]:

- *Neuroendocrine tumours* G1: mitotic count <2/10 high power fields and/or ≤ 2% Ki-67 index
- *Neuroendocrine tumours* G2: mitotic count 2-20/10 high power fields and/or 3-20% Ki-67 index
- *Neuroendocrine carcinoma* G3: mitotic count >20/10 high power fields and/or > 20% Ki-67 index → small-cell carcinoma/large cell neuroendocrine carcinoma

Well-differentiated NETs, G1 and G2, were characterized by small and monomorphic cells arranged in islets or trabeculae with granular chromatin pattern. On the other hand, poorly differentiated forms (G3) were often characterized as sheets of pleomorphic cells with extensive necrosis [76]. Moreover, in the 2010 WHO/AJCC classification of tumours of the digestive tract, the new term MANEC was also introduced in order to indicate mixed exocrine and endocrine tumours. Furthermore, in 2010 classification, the staging system based on variable and progressive tumour size/local invasion (T1- T4), nodal involvement (N0-N1), and distant metastases (M0-M1) was devised for all anatomic sites [43].

The last available classification for NENs of the digestive system was published in 2019 by WHO (table 1). The general principles of this new classification were based on a consensus meeting in Lyon. This classification divide NENs into NET and NEC according to their molecular differences. Indeed, well- differentiated NETs are characterized by mutations in MEN1, DAXX, and ATRX while NECs by TP53 or RB1 mutations [24, 77].

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

[a]Mitotic rates are to be expressed as the number of mitoses/2 mm^2 (equalling 10 high-power fields at 40× magnification and an ocular field diameter of 0.5 mm) as determined by counting in 50 fields of 0.2 mm^2 (i.e. in a total area of 10 mm^2); the Ki-67 proliferation index value is determined by counting at least 500 cells in the regions of highest labelling (hotspots), which are identified at scanning magnification; the final grade is based on whichever of the two proliferation indexes places the neoplasm in the higher grade category. [b] Poorly differentiated NECs are not formally graded but are considered high-grade by definition. [c] In most MiNENs, both the neuroendocrine and non-neuroendocrine components are poorly differentiated, and the neuroendocrine component has proliferation indexes in the same range as other NECs, but this conceptual category allows for the possibility that one or both components may be well differentiated; when feasible, each component should therefore be graded separately.

Table 1: *Classification and grading criteria for NENs of the GI tract and hepatopancreatobiliary organs* [77].

1.2.2 PANCREATIC NEUROENDOCRINE NEOPLASMS

pNENs are malignancies arising from the Islets of Langerhans and, therefore, are different from exocrine pancreas cancer [78]. pNENs are generally slow growing with OS rates of 33% at 5 years, 17% at 10 years and 10% at 20 years. However, patients with a fast growing tumour (G3 stage) rarely survive 1 year [56].

pNENs represent 7% of all NENs and ~2% of all pancreatic neoplasms with an incidence of 1–2 per

100,000 people per year [79]. pNENs show no significant sex difference and occur in a wide age range, with the highest incidence found in patients aged 30-60 years; also in this case the incidence of pNENs have increased over the past 40 years [56]. Although the major part of pNENs are sporadic, they may develop in association with familial syndromes. Approximately 30–80% of patients with MEN1, up to 20% of patients with VHL syndrome, 10% of patients with neurofibromatosis and 1% of patients with tuberous sclerosis will develop pNENs [80]. In pNENs MEN1 gene is somatically inactivated in 45% of the cases whereas DAXX and ATRX genes are mutated in ~ 45% of the cases. mTOR pathway proteins, such as TSC2 and PIK3CA, have also been reported to be altered in 15% of cases. Other frequent mutated genes are involved in cell cycle progression as TP53 and RB1 and are related to high tumour aggressiveness [81]. The prognosis of pNENs depends on clinicopathological factors, such as tumour size, Ki-67, and differentiation. Indeed, the 2010 WHO classification of pNENs was based on Ki-67 expression and mitotic counts mirroring the one for GEP-NENs. pNENs were therefore divided into 2 groups [82]:

- Well-differentiated NENs, called pNETs (G1 and G2)
- Poorly-differentiated NENs, called pNECs (G3)

This classification has proved during years to be functional but some evidences appeared during latest years had to be taken in account. Therefore, in 2017 another classification has been proposed for pNETs by WHO. This new classification followed the eight edition of AJCC/Union of International Cancer Control (UICC) TNM staging system that corresponds to the ENETS TNM classification [44, 83]. Evidences that have lead to this new classification were several. The first and the more important was that some pNETs have been found to display a Ki-67 proliferation index > 20%. According to the previous classification these malignancies would have been included in G3 group [56, 83]. However, although these malignancies appeared to have a worse prognosis than G2 group, their behaviour was still less aggressive than pNECs. Thanks to these data, the new category of well-differentiated tumour called G3 was included into classification [84]. This group showed a

well-differentiated histological pattern characterized by low p53 levels, absence of RB loss and with a Ki-67 proliferation index >20%. In 2017 classification was not specified an upper limit for mitotic rate or proliferation index, however, usually their mitotic rate and Ki67 index do not exceed 20/10 HPF and 55% respectively. pNETs, including pNETs G3, usually have some common characteristics: they weakly express p53 (in <20% of the tumour cells), don't show RB1 loss and, instead, often show positivity for SSTR2A and exhibit loss of DAXX or ATRX expression in approximately 45% of cases. pNECs retained their Ki-67 lower cut off point of 20% but with a poorly differentiated histology (either for small or large cell carcinomas) often accompanied by high p53 expression , lack or RB and retain of DAXX and ARTX expression [(85)]. Another change introduced by 2017 WHO classification for pNENs regarded the terminology used for the mixed neoplasms. The new term MiNEN have been introduced in order to replace MANEC since occasionally the mixed forms can contain a non-neuroendocrine component different from adenocarcinoma [(86)]. The current pNENs classification is the one proposed by WHO in 2019 for GI tract that have few differences from the one proposed in 2017 [(77)].

In general, an accurate classification accompanied by a correct understanding of disease staging is essential for the appropriate approach to therapy determination [(87)]. pNEN diagnosis is mostly influenced by hormonal hypersecretion that usually leads to early diagnosis due to the manifestation of clinical symptoms [(88)]. However, since up to 85% of patients do not display a specific syndrome, pNEN are often discovered incidentally at advanced stages or due to the presence of local compressive diseases [(79)]. Indeed, pNENs can be divided into two groups, functioning or not-functioning, according to their hormonal secretion. Since poorly differentiated forms rarely produce hormones, this differentiation usually accounts for well-differentiated pNET and not pNEC.

Non-functioning (non-syndromic) pNETs are not associated with a clinical hormone hypersecretion but may secrete peptide hormones and biogenic substances at insufficient levels to cause

symptoms or give a clinical syndrome [89]. Symptoms related to not-functioning pNETs are mostly due to local spread such as abdominal pain, nausea and duodenal/biliary obstruction. However, the majority of not-functioning pNETs remain clinically silent coming to clinical attention only when metastases are present [90]. Non-functioning pNET measuring < 5 mm are called pancreatic neuroendocrine microadenomas and are considered as biologically benign. The occurrence of several microadenomas has been observed in MEN1 syndrome and these malignancies during years have been associated with several hereditary syndromes [91]. On the other hand, there are not known etiological factors for sporadic not-functioning pNETs. Hystorically, non-functioning pNET were reported to constituite only 1/3 of all pNET but, however, their relative frequency has increased to 70/80% of all pNETs. Their reported incidence is approximately 0,2-2 cases every 100 000 person-years [56].

Functioning pNETs are often associated with clinical syndromes caused by abnormal hormone secretion and are categorized in several groups:

- Insulinomas: These malignancies have an estimated annual incidence of 0.4 cases per 100 000 population and represent the most common functioning pNETs [56]. ~ 90% of insulinomas occur sporadically and display a benign behaviour whereas 10% are associated to MEN1 syndrome [92]. Insulinomas are functioning well differentiate pNETs composed by insulin-producing and pro-insulin producing cells. Insulinomas cause an uncontrolled insulin secretion and, therefore, hypoglycaemic syndrome leading to adrenergic and neuroglycopenic symptoms. Adrenergic symptoms include palpitation and tremor while cholinergic symptoms include sweating, hunger and paraesthesia [93]. Delay in diagnosis is mostly due to the fact that symptoms are not specific [94]. Biochemical diagnosis of insulinoma requires demonstration of inappropriately elevated insulin, C- peptide, and pro-insulin levels in the presence of low serum glucose. Once that the biochemical diagnosis of insulinoma is secure, the tumour is commonly localised radiologically [92].

- Gastrinomas: gastrin is a peptide hormone able to promote the release of gastrin stimulating GI tract motility. Gastrinoma is a functioning well-differentiated pNET composed of gastrin-producing cells (G cells) with uncontrolled gastrin secretion causing Zoollinger-Ellison syndrome (ZES) [(95)]. Gastrinomas have an estimated annual incidence of 0.05-0.2 cases per 100 000 population but, since G cells are spread in all GI tract, those arising in the pancreas are rare [(56)]. The syndrome can arise either sporadically (approximately 70– 75%) or in association with MEN1 syndrome. Symptoms include duodenal ulcer, gastro-oesophageal reflux, diarrhoea, nausea, vomit and abdominal pain caused by gastric acid hypersecretion [(96)]. The diagnosis of gastrinoma is made when serum gastrin levels are inappropriately elevated in the setting of excessive gastric acid production. However, regardless of the diagnostic test used for biochemical confirmation, localization of the tumour is mandatory to assess the chances for surgical treatment [(97)].
- Glucagonomas: glucagon is a peptide hormone secreted by pancreatic α cells with an opposite action compared to insulin [(98)]. Glucagonoma is an extremely rare functioning well-differentiated pNET composed of glucagon producing and preproglucagon-derived peptide producing cells, with uncontrolled glucagon secretion causing glucagonoma syndrome [(56)]. Only the 20% of glucagonomas occur in association with MEN1 and there are not known etiological factors for sporadic solitary glucagonomas. Glucagonomas are often large and malignant tumours that account for 1-2% of all pNETs and represent the fourth most common functioning pNETs after insulinoma, gastrinoma and VIPoma. Glucagonoma annual incidence is approximately 1 case per 20 million population. The average patient age at diagnosis is 52.5 years and the male-to-female ratio is 1:1 [(56)]. Since glucagon stimulates glycogenolysis and gluconeogenesis, the symptoms comprise diabetes mellitus, weight loss, muscle wasting, impaired glucose tolerance and necrolytic migratory erythema. This last symptom has a very distinctive appearance, with itchy rash on the perineum, thighs, and

distal extremities prone to secondary infections [99]. Markedly elevated glucagon levels are a specific diagnostic component since elevated hormone plasma levels are generally observed only in glucagon-producing pNETs [100].

- Somatostatinomas: somatostatin is a peptide hormone whose synthesis mainly occurs centrally, in the hypothalamus, and peripherally in the pancreatic δ cells, the gastric antral D cells and the APUD cells [101]. Somatostatinoma is a functioning well-differentiated pNET composed of somatostatin-producing cells with uncontrolled somatostatin secretion causing somatostatinoma syndrome. Somatostatinomas are extremely rare and account for less then 1% of functioning pNET and their annual incidence has been estimated to be 1 case per 40 million population [56]. These malignancies are more common in women with an average patient age at diagnosis of 55 years. Somatostatinomas aetiology is mostly unknown even if some cases were associated with MEN1 syndrome, NF1 syndrome and VHL syndrome [102]. The most important syndrome symptoms include diabetes mellitus, diarrhoea, cholelithiasis, weight loss and hypochlorhydria. Since these symptoms are nonspecific, somatostatinomas are often detected only in later disease stages thanks to metastases with clinical manifestations. Somatostatinoma diagnosis requires careful clinical and laboratory assessment since Somatostatin-secreting cells can be found also in medullary carcinoma of the thyroid, pheochromocytoma and paraganglioma [103].
- VIPomas: Vasoactive intestinal peptide (VIP) is a peptide hormone that is mostly involved in vasodilation. VIPoma is a functioning well differentiated pNET composed by VIP-producing and other hormone-like substance-producing cells, with uncontrolled VIP secretion [104]. VIP production and secretion occur in several tissues as gut and pancreas as well as in the brain and the supra-chiasmatic hypothalamic nuclei. Pancreatic VIPomas represents ~ the 6% if functioning and 1,7% of all pNETs with a reported annual incidence of 0,05-0,2 cases for 1 million population [56]. There are not known causes for a VIPoma

development, there are only rare cases documented in which the malignancy has been associated with MEN1 syndrome. The clinical features of VIPomas include stimulation of intestinal secretion, facial flushing, inhibition of gastric acid secretion, stimulation of glycogenolysis, and hypercalcemia [(105)]. The most important symptom, that is also giving WDHHA name to the VIPomas related syndrome, is watery diarrhoea that occurs in 100% of the cases [(106)]. Laboratory assessment showing elevated serum level of VIP is usually enough to diagnose VIPomas [(92)].

1.3 NEUROENDOCRINE NEOPLASMS OF THE LUNG

Neuroendocrine tumours of the lung are a heterogeneous family of neoplasms ranging from extremely aggressive tumours with very poor prognosis to quite indolent lesions with long-term life expectancy [(107)]. Lung NENs account for approximately 20-30% of all NENs and 1-2% of all lung malignancies in adults. Pulmonary NENs are rare tumours with an age-adjusted incidence rate ranging from 0.2 to 2/100 000 population/year in both US and European countries [(38, 108)]. A better knowledge of pulmonary NENs as well as the implementation of radiological/immunohistochemical techniques have probably represented an important factor in the 6% per year observed increase in the last 30 years. In order to improve diagnostic recognition, the 2015 WHO classification has grouped pulmonary NENs into the unique box of "neoplasms" including all the four histologic variants of lung NENs: typical carcinoid (TC), atypical carcinoid (AC), large cell neuroendocrine carcinoma (LCNEC) and small cell lung carcinoma (SCLC). TC and AC largely share the same well-differentiated morphology and a similar genotype (G1/G2) while SCLC and LCNEC are both high grade NECs corresponding to the poorly differentiated neuroendocrine neoplasms (G3) [(109)]. TC and AC are close to the normal NE elements present in the respiratory mucosa and defining criteria of these tumours include organoid growth pattern, absent or focal punctate necrosis, up to 10 mitoses per 2 mm^2 and a consistent labelling for

IHC markers. On the opposite, SCLC and LCNEC show trabecular growth pattern, extensive necrosis, mitotic count higher than 10 mitoses per 2 mm^2 with no theoretical upper limit and uneven cell decoration for markers [107]. Lung NENs mostly occurs in the fourth to sixth decade of life with a median age at diagnosis of 64 years; however, an earlier age at diagnosis has been reported for TC. TCs are the most common primary lung neoplasms in children and late adolescents having a greater incidence in female over male. These neoplasms are usually sporadic but can also occur in the setting of MEN1 syndrome with a predominance of TC forms [110]. Concerning environmental risk factors associated with pulmonary NENs there are contrasting evidences. A family history of cancer has been reported from an US case-control as the main prognostic factor for lung NENs whereas a Swedish study reported a slightly increased risk in pulmonary NEC development in patients with a family history of NENs [111]. Moreover, cigarettes smoking represent another controversial point: it is well known that SCLC and LCNEC are associated with cigarettes smoking but there are not clear evidences regarding TC and AC [112].

1.3.1 CLASSIFICATION OF NEUROENDOCRINE NEOPLASMS OF THE LUNG

The classification of neuroendocrine malignancies has been an evolving process started in 1972, when atypical carcinoid was initially defined according to the number of mitoses per HPF, the presence of necrosis, increased cellularity with disorganization, nuclear pleomorphism, hyperchromatism, and an abnormal nuclear–to–cytoplasmic ratio [113]. In 1991, Travis et al proposed 4 categories of neuroendocrine lung tumours: TC, AC, LCNEC and SCNEC [114]. Following these classifications, in 2004 WHO proposed another classification that was based on common microscopic, immunologic and morphologic features identifiable by light microscopy. The classic carcinoids include low-grade TC and intermediate-grade AC whereas the high-grade malignancies include LCNEC and SCNEC. In the same years, pathologists proposed another

classification for neuroendocrine tumours of the lung in order to clarify the nomenclature of these malignancies. Rather than typical versus atypical, the tumours were classified into grades [115]. G1 represented tumours formerly classified as TC, G2 represents AC or well differentiated tumours, and G3 represented the poorly differentiated tumours. G3 were further divided according to cell type [116]. According to WHO 2004 classification, the differential diagnosis among neuroendocrine neoplasms is based on the assessment of two parameters: presence/absence of necrosis and mitotic index per 2 mm^2 [117]. In 2015, the 7th edition of the WHO classification of tumours of the lung, pleura, thymus and heart was proposed without introducing Ki-67 as diagnostic criteria, classification is represented in table 2.

Differentiation	Grade	Mitotic rate	Diagnosis
Well differentiated	Low grade	<2 mitoses per 2 mm^2 AND no necrosis	TC tumor
	Intermediate grade	2–10 mitoses per 2 mm^2 or foci of necrosis	AC tumor
Poorly differentiated	High grade	$\geqslant 11$ mitoses per 2 mm^2	SCLC LCNEC

TC, typical carcinoid; AC, atypical carcinoid; SCLC, small cell lung cancer; LCNEC, large cell neuroendocrine cancer.

Table 2: *2015 WHO classification of lung NENs* [118].

In this classification the terminology "carcinoid" was maintained for well and moderate differentiated neuroendocrine tumours as well as large-cell and small-cell neuroendocrine carcinoma were maintained for high grade NENs. Grading criteria were [38, 118, 119, 120]:

- Typical Carcinoid: carcinoid morphology and < 2 mitoses / 2 mm^2, no necrosis and ≥ 0.5 cm
- Atypical carcinoid: carcinoid morphology and 2-10 mitoses / 2 mm^2 and/or necrosis
- Large-cell neuroendocrine carcinoma: neuroendocrine morphology, high mitotic rate (> 10 mitoses / 2 mm^2), necrosis, typical citology and immunoreactivity for at least one neuroendocrine marker in more than 10% of neoplastic cells
- Small-cell neuroendocrine carcinoma: small-sized cells, scant cytoplasm, nuclei with granular nuclear chromatin, high mitotic rate (> 10 mitoses / 2 mm^2) and frequent necrosis.

After 2015 classification several issues have been raised concerning its utility: the missing introduction of Ki-67 as diagnostic/prognostic meter was the crucial debated point [22]. Several studies have tried to evaluate Ki-67 in lung NENs diagnostic as similar as other organ sites but without conclusive results. Since lung NENs have markedly different prognostic implications and treatment regimens, the importance of accurate pathologic diagnosis is underscored and cannot completely adopt other sites classification [121]. However, a general consideration is that pulmonary NENs classification has been a stepwise process based on a constellation of cytological and histological traits alongside the evaluation of mitotic count and necrosis extent [107]. Moreover, in 2015 classification, there was no specific staging for pulmonary NENs, that were staged following the same indications formulated for non-neuroendocrine lung cancer [122].

1.3.2 TYPICAL AND ATYPICAL BRONCHO-PULMONARY NEOPLASMS OF THE LUNG

Typical and atypical broncho-pulmonary neuroendocrine neoplasms (BP-NENs), also called bronchial carcinoids (BC), represent well differentiated malignancies developed in the surface of bronchial glandular epithelium. This is a small tumour group representing from 0.4% to 3% of lung resected pulmonary tumours and about 25% of all carcinoids. These tumours are considered indolent but, especially during latest years, all BP-NENs started to be classified as malignant since can locally infiltrate and metastasize [123, 124]. TC tumours are more frequent than AC and both differ in location, size, presence of necrosis and mitotic activity. AC tumours are more common in men and often occur in an older age than TC (average age about 57 years). About 30% of patients with lung carcinoid initially do not display any clinical signs and common reported symptoms include cough (32%), hemoptysis (26%) and pneumonia (24%) [125]. BCs can also be related to CS that has been reported in about 2/3% of patients with the occurrence of Cushing’s syndrome due to an ectopic production of adrenocorticotropic hormone [126]. The aetiology for this rare type of

NENs is not well known and its occurrence is frequently reported as sporadic. However, Approximately 5% to 10% of cases have been associated with MEN1 syndrome [127].

1.4 DIAGNOSIS OF NEUROENDOCRINE NEOPLASMS

The clinical presentation of NENs varies according to different factors such as site of origin, tumour size, presence or absence of metastatic spread, eventual association with hereditary syndromes, eventual hormones hyperproduction and related syndromes [128]. Thanks to these variants usually diagnosis occurs at an advanced stage with distant metastases (usually hepatic) in about 50-85% of patients [129, 130]. Diagnosis is mostly influenced by hormonal hyper secretion that usually leads to early malignancy discovery [88]. However NENs are often discovered incidentally, during surgery or radiological assessment, at advanced stages [79]. Another component that can be important in an early diagnosis is the presence of local compressive symptoms due to tumour growth [74]. NENs initial diagnostic approach includes histological examination, which is always required before every therapeutic decision in order to identify tumour stage. Repetitive biopsies, analysis of immunohistochemical markers, detailed histological analysis, assessment of the primary tumour and extension of extrahepatic spread by imaging and valuation of general tumour markers are the usual investigations required for a correct diagnosis [131]. A representation of diagnosis process is represented in figure 1 [132].

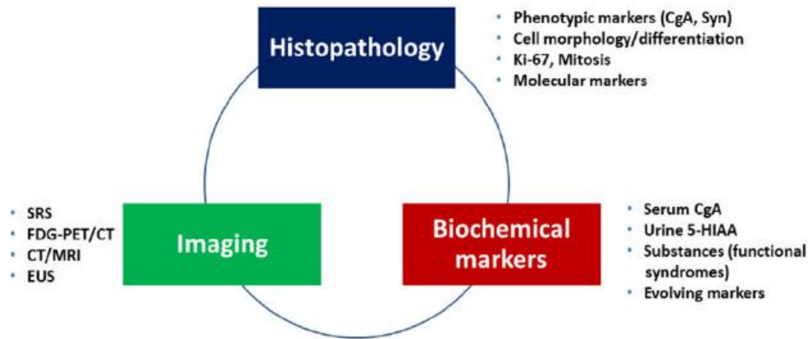

Figure 1: *Diagnostic tools for NEN classification* [132].

1.4.1 CIRCULATING BIOMARKERS

Various cell types, able to secrete products as peptides and biogenic amines, compose the neuroendocrine cell system. Neuroendocrine cell cytoplasm is occupied by a large number of secretory granules, which constitute the storage site of secretory products [133]. After specific stimulation, granules are translocated to the cell membrane and their content released by exocytosis. Sometimes these products may be used as markers for the diagnosis and follow-up of treatments and, therefore, have prognostic implications. Indeed, the presence of these secretory products in the serum can be exploited diagnostically as tumour markers for NENs and are usually divided into general markers and specific markers, depending on the cell type involved [134]. Overall, biomarkers are essential since they can facilitate the prediction, cause, diagnosis, progression, regression, or treatment outcome of the disease. The major problem related with biomarkers is the identification of “good biomarkers”. This means biomarkers uniquely found in the malignant tissue generating a positive signal that can be measured without be confused with the ‘noise’ from normal tissues or other non-malignant pathologies. The identification of correct range values for biomarkers reflective of a specific neoplasia, is therefore required for a correct biomarkers evaluation. Otherwise the risk is to obtain false positive results [135]. Unfortunately, most of the currently

used circulating neuroendocrine markers are relatively nonspecific and can be influenced by tumour growth, be a consequence of chemo/radio-metabolic treatments or other factors illustrated in table 3 [136]. For a correct diagnosis it is really important to understand whether signs and symptoms noticed during routine clinical examinations could be suggestive of NENs, and therefore investigate biomarkers accordingly to clinical suspicion. Indeed, in NENs case, biomarkers should never be considered "screening" tools but additional indicators after other clinical manifestation [92]. Usually neuroendocrine markers are divided in 2 main categories: general markers and specific markers.

General markers:

- Chromogranins (Cg) → CgA is the most frequently used biomarker for the diagnosis and follow-up of NENs [137]. It ss a glycoprotein secreted by neurons and neuroendocrine cells and, like all the other proteins belonging to the granins family, it is a precursor of biologically active substances as pancreastatin, catestatin, and vasostatins I and II. CgA is exclusively expressed in the secretory dense core granules of most normal and neoplastic neuroendocrine cells and, upon stimulation, is co-released with peptide hormones and neuropeptides [138]. Elevated circulating levels of CgA have been demonstrated in patients with both functionally and non-functionally NENs and are considered a marker of bad prognosis in both ileal and pancreatic NENs. Three diagnostic techniques are available in detecting CgA protein levels: enzyme-linked immunosorbent assay (ELISA), immunoradiometric assay (IRMA) and radioimmunoassay (RIA). Determination of CgA by different techniques can lead to significant differences in results, with several effects on sensitivity and specificity [139]. The sensitivity of the CgA have been proved to be around 60–80% and is dependent of primary site, grade and status of the disease. However, caution it's always suggested in CgA interpretation since several factors can increase its levels such as drugs, steroids as well as oncological and non-oncological conditions. Increased CgA level has also been reported in pancreatic adenocarcinoma hepatocellular cancer and a

plethora of endocrine diseases such as hyperthyroidism and hyperparathyroidism [140].

	Biomarker	Increased by ...	Reduced by ...
Non-specific markers	Chromogranin A	Breast cancer, prostate cancer, ovarian cancer, hepatocarcinoma, pancreatic adenocarcinoma, colon cancer, kidney failure, heart failure, hyperthyroidism, hyperparathyroidism, chronic obstructive broncho–pulmonary disease, gastrointestinal pathologies, steroids, proton pump inhibitors	?
	Neuron-Specific Enolase	Thyroid cancer, prostate carcinoma, neuroblastoma, and small cell lung carcinoma	Neuronal damage
	Pancreatic Polypeptide	Physical exercise, hypoglycemia, food intake	Somatostatin, hyperglycemia
	Human Chorionic Gonadotropin	Pituitary tumors, pregnancy	?
	α-fetoprotein	Liver injury, pregnancy	?
Specific markers	Serotonin	Tryptophan-rich drugs (diazepam, ephedrine, phenobarbital, phentolamine ...) and foods (peanuts, bananas, avocados, chocolate, vanilla, coffee, tea ...), nicotine, malabsorption, celiac disease	Ethanol, ACTH, streptozocin, acetylsalicylic acid, heparin, MAO inhibitors, renal failure, hemodialysis
	Gastrin	Hypochlorhydria or achlorhydria, chronic atrophic gastritis, renal failure, *H. pylori* infection	Acetylsalicylic acid, levoDOPA
	Insulin	Hyperglycemia (including factitious and sulfonylurea-induced hypoglycemia), insulin resistance, insulinoma	Hypoglycemia, Type 1 Diabetes Mellitus, noradrenaline, fasting, glucagon
	Glucagon	Hypoglycemia, adrenaline, arginine	Hyperglycemia, somatostatin, insulin
	Somatostatin	?	?
	Vasoactive Intestinal Peptide	Bowel inflammation and ischemia	?

Table 3: *Factors interfering with serum biomarkers assays* [92].

- Pancreatic polypeptide (PP) → PP is a protein mostly secreted by pancreatic islet cells, whose function is largely unknown even if it is believed that can influence GI secretion. Due to its low sensitivity and specificity (63% and 81%, respectively), PP is generally considered a NENs marker with low usefulness in clinical practice. Moreover, PP serum concentration can be increased by many factors as physical exercise, hypoglycemia and food intake. A significant improvement in sensitivity can be obtained by the combination of PP detection with another marker, most commonly CgA. However, its role as prognostic biomarker is still largely unknown [141, 142].
- Neuron-specific enolase (NSE)→ NSE is a neuron-specific isomer of the enolase

enzyme, found in neurons and neuroendocrine cells [143]. This enzyme is used as a biomarker but, since only 30 to 50% of NENs secrete NSE, its assessment alone is rarely adequate for diagnostic purposes. Therefore, its diagnostic sensitivity in GEP-NENs is low (32–47%). Moreover, erythrocytes present a large amount of NSE and can cause false positive results [138]. Indeed, NSE serum levels are also associated with multiple other factors such as thyroid cancer, prostate carcinoma, neuroblastoma, and SCLC. NSE and CgA assessment can improve the reliability of diagnostic process providing further proof of the presence of a NEN. However, given the non-specific nature of both markers, these tests provide little information concerning the localization of the primary tumour and its development stage [92].

- Human chorionic gonadotropin subunits (hCG)→ hCG is a heterodimeric glycoprotein physiologically synthesized during pregnancy by the placenta. This protein is composed by two different subunits: α subunit, which is shared with pituitary hormones, and β subunit, unique subunit characteristic of the protein [144]. A different hCG expression pattern has been identified for a long time in different endocrine and non-endocrine tumours. Indeed, tumours cells often lack the mechanisms to pair the two subunits and increased expression of α subunit is commonly found in pituitary tumours and NENs whereas pancreatic tumours often display increased secretion of β subunit. However, hCG is rarely used in everyday clinical practice for NENs and, even if an increase of hCG in association with alpha-fetoprotein (AFP) has proved to have prognostic value, its assessment is generally not recommended in NENs [145].

Specific markers: in neuroendocrine diseases there are several families of secretory proteins that have been found in high concentrations and are therefore used in the identification of NENs in the pathology laboratory [146]. Beyond general markers there is another category that is composed by different specific hormones secerned by NENs such as serotonin, insulin, glucagon and other specific products. These factors are generally signals of a specific malignancy. A high blood value

of these hormones, in association with the analysis of other biomarkers, can represent an important factor in diagnosis process and is therefore commonly performed in clinical practice. This is particularly relevant especially for GI-NENs since are the category majorly related to specific markers [147].

Over the most common used biomarkers there are also new factors that are acquiring more consensus day by day. One of the most recent is the paraneoplastic antigen ma2 (PNMA-2), an autoantibody produced by the small intestine NENs, which has been associated with a poor PFS. Moreover, one of the greater recent areas of interest is the detection of circulating tumour cells (CTCs) [148]. Indeed, it has been reported that CTCs have an important potential role for early diagnosis and prognosis since they can be found before the primary tumour is detected and provide minimally invasive access to tumour tissue. Most CTC-identifying assays involve the use of antibodies against epithelial markers, e.g. EpCAM, the epithelial cell adhesion molecule [149]. CTCs have been reported to be detectable in both migdut (43%) and pancreatic NENs; however, no correlation was found with Ki-67 and serum CgA, respectively. Moreover, no association was observed between an eventual decrease in the number of CTCs after somatostatin treatments and other therapies. Therefore, even if CTCs technology and concept have had an attractive value proposition, the actual technology platform does not indicate that they can be used in its current form as an effective biomarker for NENs [135]. Another interesting biomarker category is represented by microRNAs (miRNAs). miRNAs are a class of small (19–25 nucleotides) noncoding RNAs that acts as posttranscriptional regulators in several biological processes. miRNAs levels have been reported to be disregulated in several malignancies and, therefore, miRNAs are considered to have potential as both biomarkers and therapeutic agents. Every NENs subtypes have shown to have a peculiar miRNAs expression pattern. For example, in pNENs several miRNAs deregolations have been reported such as upregulation of miR-103, miR-193b and downregulation of miR-1290 [150]. However, in 2013, the American Association for Clinical Chemistry reported that

the detection/quantification of miRNAs were not robust, rapid, simple, accurate, reproducible nor inexpensive and that there was a low correlation between the several platforms used in different laboratories. Overall, the clinical application of miRNAs as biomarkers has demonstrated to be challenging especially for a lack of standardization [(135)].

In conclusion, circulating biomarkers analysis offers a useful diagnostic tool in association with radiology and tissue pathology for NENs. However, these biomarkers are more reliable when used to monitor disease progression, response to treatment, and for an earlier indication of recurrence after treatment [(146)].

1.4.2 DIAGNOSTIC IMAGING

A very important area for biomarkers development is molecular imaging. Since few patients achieve tumour shrinkage, it is becoming clear that conventional assessment of tumour response by RECIST criteria is not very appropriate for NENs. Instead, there is a large group of patients in which drugs prolong tumour stabilization and, in this scenario, there is a high interest in the development of molecular imaging able to detect antiproliferative drug effects [(148)]. In NENs diagnosis and management, the anatomic imaging plays a crucial role largely due to its ability in providing anatomical information for surgical planning. Several morphological (anatomical) and functional (molecular) imaging techniques are available in NENs management and are often used in combination. In order to identify tumour stage the two main diagnostic methods are CT (computed tomography) or MRI (magnetic resonance imaging) in combination with somatostatin receptor imaging performed by scintigraphy or PET (positron emission tomography) [(151)]. The presence of somatostatin receptors on many neuroendocrine tumour cells was the main reason for the development of SRS. Moreover, beside receptors, the important metabolic activity of specific biochemical pathways provided possibilities for molecular nuclear medicine imaging. NENs

have unique characteristics that have led to the development of interesting new diagnostic methods over the last years, both in receptor imaging and metabolic imaging [152]. CT represents the mainstay for abdominal NENs imaging and is generally readily available with the advantage, when in comparison with ultra sounds analysis, of a wider field of view. It is, therefore, suited for detection of nodal and metastatic disease. In terms of lesion detection, the sensitivity with CT increases proportionately with lesion size. Indeed, in gastric NENs have been demonstrated that lesions larger than 1 cm in diameter were detected with an increased frequency. Moreover, since it was observed that improvements in CT have led to an increase in lesion detection, CT confirmed to be over years one of the most important imaging techniques [153]. MRI it's another imaging technique commonly use in NENs imaging. This technology may be used to confirm a CT finding or to localize a suspected lesion that has not been clarified with CT. MRI offers greater sensitivity and specificity in the detection of both pancreatic mass and liver metastases, making it appropriate for surgical planning, particularly for the assessment of smaller lesions [154]. Other imaging techniques employ specific isotopes such as somatostatin receptor scintigraphy (Octreoscan), MIBG- scintigraphy, or PET with a large variety of tracers (i.e., ^{11}C- 5HTP, ^{11}C-Dopamine, ^{18}F-FDG, ^{68}G-DOTA-Octreotide, or ^{99}Tc EDDA-HYNIC-Octreotide). These isotopes are currently used in the clinic to identify tumours that express somatostatin receptors, have a high hormone/peptide production or have simply a high proliferative activity [148]. PET is used in the diagnosis of poorly differentiated disease or well-differentiated disease with high proliferation rates or rapid clinical progression whereas somatostatin-receptor scintigraphy (SRS) is a functional imaging method that measures the binding of radiolabeled somatostatin analogs to somatostatin receptors on the surface of NENs. SRS showed a reported sensitivity of ~90% and specificity of 80% and is recommended for all patients with suspected NENs but it's still not completely clarified its role in disease follow-up assessment and surveillance [155].

1.5 NEUROENDOCRINE NEOPLASMS MANAGEMENT

Since NENs diagnosis is often delayed for months to years, even in functional tumours, approximately 75% of patients with NENs display metastatic disease at presentation, most commonly in the liver and less frequently in bones. This problematic is mostly due to the nonspecific and intermittent nature of NENs symptoms [(156)]. Multiple options are available for the management of patients with advanced, metastatic pancreatic NENs, including surgical resection, liver-directed therapies, and systemic therapy. An overview of NENs investigation and treatment is illustrated in figure 2 [(10)]. Therapies goal concerns the improvement of symptoms related to hormone hypersecretion, disease progression slow-down and survival's improvement [(157)]. The initial phase of evaluation is the identification of tumours functionality: when functional tumours are suspected biochemical testing should be employed to confirm the diagnosis. Anatomic localization represents the second phase of evaluation. Multiphasic CT scan is typically the imaging modality of choice but, MRI may be used for some patients. Depending on these results, SRS can be helpful and is typically used to identify metastatic disease. Endoscopic ultrasonography may be really useful, especially for pNENs, since allows high-resolution image and provides the ability to perform biopsies. Finally, visceral arteriography and selective intra-arterial stimulation may be helpful in localizing occult functional tumours. Once this evaluation has been completed, patients with early-stage disease should require a surgical consultation.

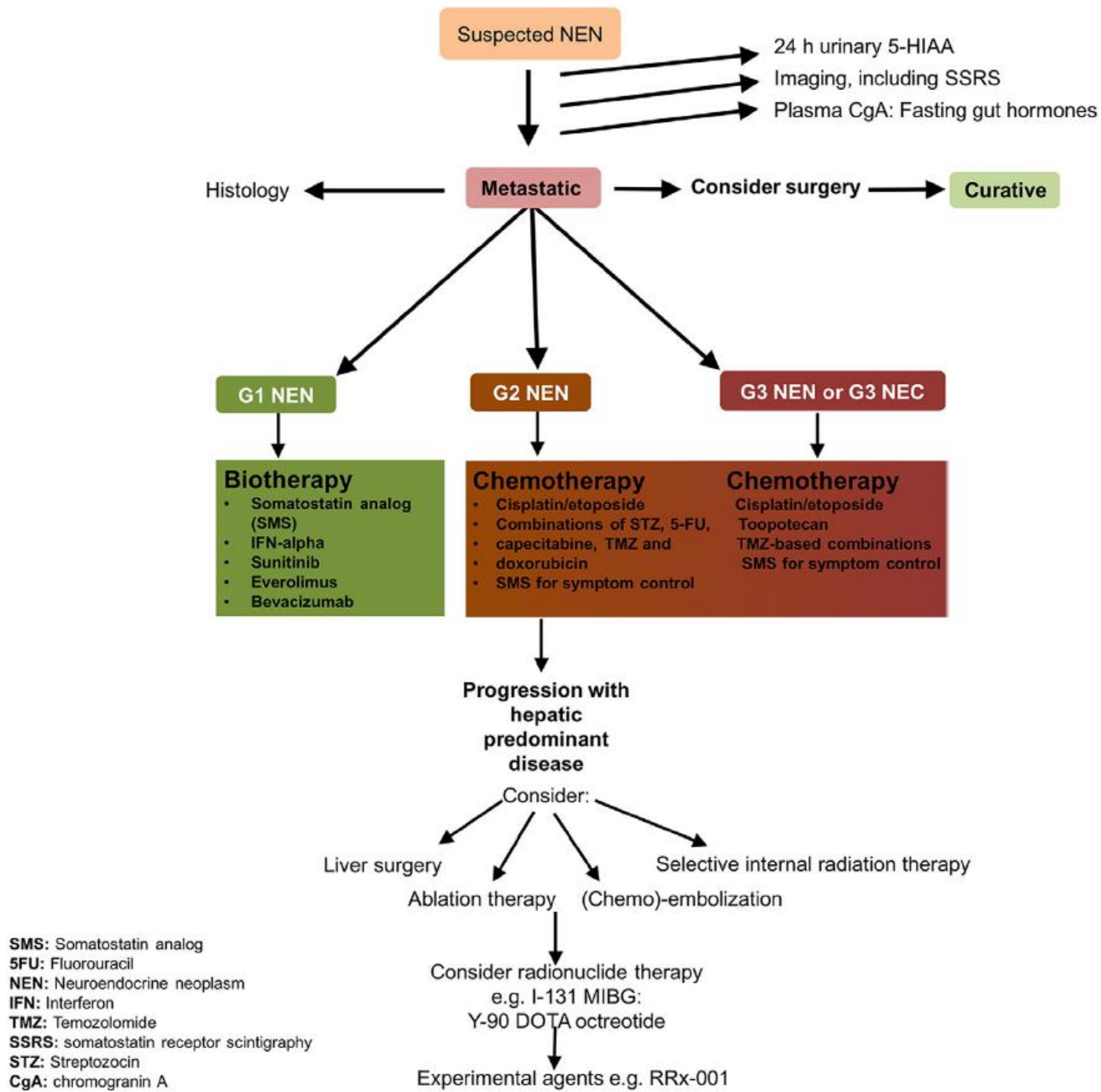

Figure 2: *NENs investigation and treatment overview* [10].

For patients with recurrent and/or metastatic disease, cytoreductive surgery is a key modality, but sometimes may be supplemented with other therapies. For those patients who are not candidates for surgical intervention, locoregional or systemic therapies should be employed [158, 159]. Due to the disease heterogeneity in terms of clinical behaviour and origin, a multidisciplinary approach is required. Surgical resection remains the most effective treatment and the elective strategy but, since > 50% of NENs are not resectable at diagnosis, alternative approaches are required. Systemic therapy options include the use of somatostatin analogue, cytotoxic chemotherapy, radionuclide therapy and new molecular targeted agents [33, 160].

1.5.1 SURGERY AND CHEMOTHERAPY

Complete surgical resection represents the first-line and the only potentially curative treatment of primary NENs, regardless of their origin. However, surgical approach is influenced by lesion size and location, disease stage, and patient's symptoms [161]. When NENs are diagnosed at an early stage, surgical resection is often curative but, unfortunately, usually it is not possible or decisive for patients with metastatic disease. In these patients, palliative surgery to remove or debulk the primary tumour is recommended, but it depends on its location and involvement of surrounding tissues. In order to achieve symptom control, in case of functioning NENs, at least 90% malignancy resection is required. Resection is also helpful in the increase of systemic therapies efficacy [162]. Appropriate candidates who undergo to surgery have a significant survival advantage compared with those who do not. The correct surgical management must be individualized for patients and based on their particular tumour site of origin and classification [90]. Aggressive surgical therapy is recommended for metastatic disease when all diseases can be safely removed. Moreover, for patients with hepatic metastases, liver transplantation is recommended. Transarterial embolization may also be helpful in the treatment of patients with hepatic metastases not amenable to surgery.

Radiofrequency ablation (RFA) and internal radiotherapy of unresectable liver metastases are other common practises but contraindications are several with a high failure risk [158]. In addition to surgery and local ablation therapy, patients with metastatic NENs should also receive systemic therapy to control hormonal symptoms and limit tumour progression. Chemotherapy may play an adjuvant role and is indicated before and/or after surgery but can also be, in some cases, the only treatment. Chemotherapy has still a role in NENs management, particularly in those of pancreatic origin or with high proliferative index [163]. However, in the majority of cases, the use of single chemotherapeutic agents (doxorubicin, 5-fluorouracil, dacarbazine, cisplatin, carboplatin, etoposide, streptozocin) has demonstrated to lead to little beneficial effects in reducing the tumour mass or in symptoms control. Chemotherapy regimens based on Streptozocin combinations are the most

used in NENs. This drug has shown a good effect once in combination with other agents, such as 5-fluorouracil, cisplatin or doxorubicin especially in patients with pNENs. Another drug that has shown good effects in pNENs but has never been approved for the treatment is Temozolomide. Temozolomide is more efficient against cells with low MGMT (DNA repair enzyme) levels and, since pNENs cells usually lack in MGMT expression, are more sensitive to drug action [148, 164]. In general, chemotherapy should be considered for patients who are symptomatic from tumour bulk or who have a rapid progressive disease but less considered for patients with a slow growing tumour with an indolent behaviour [157].

1.5.2 RADIONUCLIDE THERAPY

In cases of inoperable tumours or after surgery, another therapeutic option for patients is represented by radiation therapy. The rationale for radiation therapy use is the presence on neuroendocrine cells surface of several specific receptors, usually 7-transmembrane- domain G-protein-coupled receptors. This evidence poses the basis for a peptide receptor-targeted therapy (PPRT) using several ligands such as dopamine or VIP [165]. However, the most exploited and known ligand-receptor system in clinical practice is somatostatin. This system can be successfully exploited since somatostatin receptors are expressed in several neuroendocrine tissues and neoplasms such as pituitary adenomas, gastrointestinal and pancreatic endocrine carcinomas, bronchial and thymic neuroendocrine tumours, paragangliomas, pheochromocytomas, small cell lung cancers, medullary thyroid carcinomas, breast cancers, and malignant lymphomas [166, 167]. Thanks to the presence of somatostatin receptors on neuroendocrine cells surface, PPRT with radioactive somatostin analogues enable the treatment of primary and metastatic lesions. Structurally, radiolabeled SSAs are composed by a radionuclide isotope, a carrier molecule (generally octreotide or octreotate) and a chelator (usually DOTA or diethylenetriamine

pentaacetic acid) that binds both, stabilizing the complex [33]. Radioactive somatostatin analogues bind all the somatostatin receptors (5 isoforms) and are internalized into endosomes activating post-receptor mechanisms that are responsible for the pharmacological effect. The receptor can be recycled or entrapped into lysosomes enabling a long irradiation of tumour cells and allowing a radionuclide-based peptide diagnosis and/or therapy, depending on the radionuclide used [168]. Different radionuclides such as ^{111} Indium, ^{90} Yttrium, ^{177} Lutetium are used and DOTA^{0}-Tyr^{3} octreotate is considered the most effective agent especially in pNENs patients. This targeted radiation treatment yields an objective radiological response in up to 20–30% of NENs patients. However, NETTER-1 randomized prospective phase 3 trial has demonstrated that, in patients with metastatic and progressing small intestinal NEN, 177Lu-Dotatate therapy markedly prolongs PFS compared with high-dose octreotide alone. In addition, the same study reported a significant quality-of-life benefit in ^{177}Lu-Dotatate arm compared with octreotide. However, PRRT is usually well tolerated with self-limiting acute side effects of nausea and vomiting (attributed mainly to amino acid infusions performed during the procedure) [44, 67, 169].

1.5.3 SOMATOSTATIN ANALOGUES

Up to 90% of GEP NENs carry somatostatin receptors on the membrane and are therefore considered candidates for somatostatin analogues (SSA) based therapy. Somatostatin is a hormone that inhibits the release of neuroendocrine products, including those released from NENs. Since somatostatin has a short half-life in vivo, synthetic somatostatin analogues were developed for NENs symptoms control [168]. Although tachyphylaxis frequently may occur, these drugs are generally well tolerated and are used successfully to control tumour hypersecretion and symptoms in up to 70% of patients. Somatostatin analogues antiproliferative activity is scarce, with objective benefits encountered in <10% of patients, while disease stabilization can be achieved in more

than 60%. This result was confirmed from multiple retrospective and phase 2 studies that have investigated SSAs antitumor effects in patients with GEP-NENs. Disease stabilization was observed in approximately 40% to 60% of patients; however, rates of objective radiographic response were low (generally <5%) [33, 170, 171]. Octreotide (Sandostatin®) was the first somatostatin analogue commercially available with high affinity for $sstr_2$ and moderate affinity for $sstr_3$ and $sstr_5$. Its antitumor effects are probably due to $sstr_2$ stimulation that leads to decreased hormonal secretion, decreased growth and proliferation, increased apoptosis, inhibition of cell signalling and inhibition of protein synthesis [172]. Lanreotide (Somatuline® Depot) is another long-lasting somatostatin analogue with a similar action to Octreotide. Both these agents can be used to control clinical symptoms caused by hormonal secretions in NENs. There are 25 years of evidence that underlie how Octreotide can control symptoms as severe diarrhoea and flushing in patients with carcinoid syndrome [173]. These drugs are well tolerated, with mild adverse effects and high tolerability after sustained use. Pasireotide (SOM230) is another multireceptor-targeted analogue with high affinity for $sstr_1$, $sstr_2$, $sstr_3$, and $sstr_5$. Since this drug can bind several SSTRs it may benefit a wide patients spectrum that maybe displayed a resistance to Octreotide and Lanreotide treatment. Both the PROMID (placebo-controlled, prospective, randomized study on the effect of octreotide LAR in the control of tumour growth in patients with metastatic neuroendocrine midgut tumours) and the CLARINET (controlled study of Lanreotide antiproliferative response in neuroendocrine tumours) trials showed a statistically significant prolongation of PFS upon SSA treatment when compared to placebo [174]. On the other hand, COOPERATE-2 and phase II LUNA trials, in which Paserotide was tested in combination with the mTOR inhibitor Everolimus, had unexpected results, showing similar PFS and OS in both trial arms. In the evolution of treatment-strategies, SSAs were combined also with interferon alpha, a protein with well-known antiproliferative and anti-secretory effects [175]. Both in combination with SSA and as single treatment, interferon alpha was demonstrated to provide hormonal control and reduce clinical symptoms of CS. However, this

positive effect was restricted by severe adverse side effects. Nowadays, SSAs are profitably used in controlling symptoms and in stabilizing tumour growth in specific settings but are not enough in stopping tumour growth [176].

1.5.4 NOVEL MOLECULAR TARGETED THERAPIES

Since chemotherapy, PPRT therapy and the use of SSAs have demonstrated to be not completely effective in NENs treatment, other therapeutic options have been actively searched for. Recent studies have led to advances in the knowledge of NENs molecular mechanisms/pathways allowing the development of novel targeted therapies that are now included in daily clinical practice [177, 178]. NENs hallmarks have been defined during last years and our comprehension of genetics, epigenetics, tumourigenesis, angiogenesis and novel biomarkers has tremendously grown in the last few years. A number of promising novel molecular targets have been defined by preclinical and early clinical studies in NENs. The rationale in choosing molecular targets was the same as for SSAs: a full presence/deep involvement of targets on/in neuroendocrine cells surface/pathways [179]. A complete panel of key randomized trials for the evaluation of antiproliferative agents in NENs patients is represented in table 4 [33]. One of the main identified targets is represented by tyrosine-kinase receptors (RTKs) that have been identified as overexpressed on NENs cells surface. Moreover, RTKs were found overexpressed also in other cell types such as endothelial cells and pericytes that play an important role in the development and the maintenance of blood supply for proliferating cancer cells through the development of tumour angiogenesis. RTKs include several receptors and VEGFR (vascular endothelial growth factor receptor) was reported as major player involved in the endothelial cell survival whereas PDGFR (platelet-derived growth factor receptor) was demonstrated to be involved in pericytes functions stimulation. VEGFR and PDGFR downstream signalling also represented one of the more targeted pathway in the development

of new therapies [118,180]. The phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) and the phospho- lipaseC/protein kinase C represented the focal area of interest such as the RAS/RAF/MAPK and JAK/STAT pathways. These studies have led to the development of drugs such as Sunitinib, well-known RTKs inhibitor, and Everolimus, an mTOR inhibitor, and to their approval for NENs treatment [181]. The high NENs heterogeneity and their low incidence, once in comparison with other malignancies, have become the main limitation in new drugs development in this field. The study of new predictive biomarkers of clinical response and international multicentre efforts may be essential in order to improve NENs management in the era of molecular targeted therapy [182].

STUDY	CONTROL VS INVESTIGATIONAL ARM	NO. OF PATIENTS	POPULATION ENROLLED	MEDIAN PFS (CONTROL VS INVESTIGATIONAL ARM)	ORR (CONTROL VS INVESTIGATIONAL ARM)
pNET					
RADIANT-3	Placebo vs everolimus 10 mg	420	Progressive pNETs	4.6 mo vs 11 mo	2% vs 5%
Sunitinib	Placebo vs sunitinib 37.5 mg	171	Progressive pNETs	5.5 mo vs 11.4 mo	0% vs 9%
ECOG E2211	Temozolomide vs CAPTEM every 4 wk	144	Progressive pNETs	14.4 mo vs 22.7 mo	27.8% vs 33.3%
Non-pNET					
PROMID	Placebo vs octreotide LAR 30 mg every 4 wk	84	Treatment-naive midgut NETs	6 mo vs 14.3 mo[a]	2% vs 2%
CLARINET	Placebo vs lanreotide autogel 120 mg every 4 wk	204	Advanced, SSTR+ GEP-NETs	18 mo vs NR	NR
SWOG S0518	Interferon-α-2b 3 times per wk plus octreotide 20 mg every 3 wk vs bevacizumab 15 mg/kg plus octreotide 20 mg every 3 wk	427	Progressive NETs with poor prognostic features	15.4 mo vs 16.6 mo	4% vs 12%
RADIANT-2	Placebo plus octreotide LAR vs everolimus 10 mg plus octreotide LAR	429	Progressive functional GI/lung NETs	11.3 mo vs 16.4 mo	2% vs 2%
RADIANT-4	Placebo vs everolimus 10 mg	302		3.9 mo vs 11 mo	1% vs 2%
NETTER-1	Octreotide 60 mg every 4 wk vs 4 cycles of ^{177}Lu-DOTATATE	230	Progressive, SSTR+ midgut NETs	8.4 mo vs NR	3% vs 18%

Table 4: *panel of the key trials for the evaluation of antiproliferative agents in NENs* [33].

1.6 SUNITINIB AND TYROSINE KINASE RECEPTORS IN NEUROENDOCRINE NEOPLASMS

RTKs are proteins that act as transmembrane receptors for a series of polypeptides including growth factors, cytokines and hormones. RTKs are composed of an extracellular ligand binding domain, an intracellular tyrosine kinase domain, a transmembrane domain and an additional amino acid sequence that function as regulatory domain. When the ligand binds the receptor it induces a dimerization of the receptor and a consequent autophosphorylation. This autophosphorylation is called trans fashion meaning that one subunit of the dimer phosphorylates the opposing subunit [(183)]. RTKs, after activation, recruit intracellular signalling proteins and, therefore, promote the activation of several pathways involved in cellular proliferation and viability. VEGFR, EGFR (epidermal growth factor receptor), IGF1R (insulin-like growth factor receptor), PDGFR, KIT and RET are the most common RTKs and their overexpression on neuroendocrine cells surface have been largely demonstrated [(184, 185, 186)]. RTKs are therefore potential oncogenes that, when over activated, can lead to an abnormal cellular proliferation. Thanks to this, RTKs represent a potential target for molecular therapy. Several RTKs inhibitors have been developed with different affinities to different RTKs types. Sunitinib (multi RTKs inhibitor), Erlotinib (EGFR inhibitor), Linsitinib (IGF1R inhibitor) and Sorafenib (multi RTKs inhibitor) are only few examples of drugs that have been developed during the last years against RTKs [(187)]. Sunitinib is the most known RTKs inhibitor and is indicated for the treatment of several NENs, such as gastrointestinal stromal tumours, advanced renal cell carcinoma and pNENs [(188)].

1.6.1 TYROSINE KINASE RECEPTORS AND THEIR PATHWAYS

The most important RTKs identified as overexpressed in NENs, and in particular in GEP-NENs, are

the ones indicated above. VEGF and VEGFR are the main factors involved in one of the most important event associated with tumour growth: vascularization. Since metastatic process is the result of cellular clones systemic dissemination, angiogenesis plays a crucial role in facilitating tumour growth and in generating metastasis. Therefore, angiogenesis regulation is one on the key processes in order to stop tumour development [(189)]. VEGF is a 45-kDa homodimer that belongs to a family of six glycoproteins: VEGF-A, -B, -C, -D and -E. VEGF effects are mediated by their binding to different receptors, VEGFR-1, -2 and -3, and include vascular permeability, endothelial cell proliferation and migration, and stabilization of new blood vessels. VEGF activates multiple downstream proteins such as protein kinase C-Raf-Mek-Erk pathway, mostly involved in endothelial cell proliferation, and PI3K/AKT/mTOR pathway, involved in cells survival [(190)]. EGF and EGFR are two other growth factor/growth factor receptor highly expressed by NENs cells. EGFR overexpression has been associated with poor prognosis and resistance to therapy in several tumour types and its role in NENs pathogenesis has not been well defined [(191)]. EGFR is present in different isoforms, EGFR -1, -2, -3, -4, on NENs cells surface and its phosphorylation leads to the activation of other intracellular proteins involved in cellular proliferation, apoptosis control and angiogenesis. EGFR signalling occurs through a complex, multidimensional pathway that mostly involves Ras-Raf-mitogen activated protein kinase (MAPK) and PI3K/AKT/mTOR pathway [(192)]. IGF1 is a small peptide consisting of 70 amino acids with a molecular weight of 7649 Da. Similarly to insulin, IGF1 has an A and B chain connected by disulphide bonds and its action in cells is mediated by the binding with the correspondent receptor [(193)]. IGF1R is a member of the receptor tyrosine kinase family and activation of this receptor triggers the initiation of intracellular events, such as MAPK kinases and PI3K/AKT/mTOR pathway, resulting in proliferation, transformation and apoptosis inhibition. IGF1 plays an autocrine regulator role in carcinoid tumours and its blockage has been of special interest in NENs thanks to the well-known effect of SSAs in decreasing IGF1 levels [(148)]. PDGF is a dimeric glycoprotein that can be composed of two A

subunits (PDGF-AA), two B subunits (PDGF-BB), or one of each (PDGF-AB). This factor plays a significant role in blood vessel formation and it's a potent mitogenic agent for mesenchymal cells such as fibroblasts and osteoblasts. The overexpression of PDGF and its receptor has been observed in several malignancies and has been associated with a bad prognosis [194]. Moreover, another RTK widely express in neuroendocrine cells is RET. This receptor has key roles in cell growth, differentiation, and survival. Following ligand binding, RET signals act through multiple downstream pathways such as RAS/MEK/ERK pathway, that promotes cell cycle progression, and P13K/AKT/NF-κB pathway, that leads to increased cell motility and survival enhancing cell cycle progression. RET mutations have been associated with different malignancies and are the focus of MEN2 syndrome [195].

1.6.2 SUNITINIB AND NEUROENDOCRINE NEOPLASMS

Sunitinib is an orally active multi RTKs inhibitor approved by EMA and FDA for unresectable or metastatic pNENs with disease progression. 171 patients, who had evidence of disease progression, with well-differentiated pNENs received Sunitinib or placebo in a multinational, randomized, double-blind, placebo controlled phase III trial [196]. During the trial, Sunitinib has shown significantly prolonged PFS (11.4 months versus 5.5 months), increased overall response rate (ORR) and stable disease rate (34.9% versus 24.7%, respectively). Thanks to the higher number of deaths and adverse reaction rates in the placebo arm, the study has never completed the planned enrolment of 340 patients. Based on this study, the drug was approved for the treatment of locally advanced or metastatic pNENs in 2011 [2, 197]. However, the low response rate to Sunitinib (9.3%) indicates that clinical benefit is mainly due to disease stabilization rather than tumour shrinkage. Furthermore, Sunitinib showed to cause several side effects as diarrhoea, nausea, vomiting, fatigue and less frequent hypertension, palmar-plantar erythrodysesthesia, neutropenia, hypothyroidism and others. Currently, there are no phase 3 studies demonstrating Sunitinib benefits in non-

pancreatic NENs [2, 33, 197].

1.6.3 RESISTANCE TO SUNITINIB

After the initial enthusiasm, intrinsic and acquired resistance to Sunitinib rapidly emerged as a challenge. In fact, almost one-third of patients treated with Sunitinib were intrinsically resistant whereas two-third were initially sensitive but develop resistance after a period of treatment having a modest OS benefit [198]. A better knowledge of Sunitinib resistance mechanism of action would provide better prognostic biomarkers that could guide clinicians toward individualized therapy. Indeed, several research groups have faced this problem obtaining important results. What is well known is that a frequently exploited strategy by cancer cells to evade cell death and sustain their proliferation involves the activation of prosurvival signalling pathways. During years Sunitinib resistance has been correlated to many factors. In pNENs resistance has been associated with an increase of negative autophagy regulation (enhanced Mcl-1 stability and mTORC1 activity) and to a lysosomal dysfunction (leading to Sunitinib sequestration and hence inactivation) [198, 199]. Other studies reported the pancreatic fibrotic stroma as the main responsible of Sunitinib resistance. Indeed, *in vitro* studies demonstrated Sunitinib efficacy on cultured cells leading to the idea that the lack of effects observed *in vivo* might be due to impaired drug delivery promoted from pancreatic fibrotic barrier, rather than cells insensitivity to the drug [200]. Moreover, GIST studies demonstrated that resistance could be due to acquired secondary genetic mutations. *In vitro* data have indeed demonstrated that more resistant cells showed secondary mutations not identified at the beginning of the treatment. In RCC, evidences demonstrated that Sunitinib resistance was accompanied by evasion of antiangiogenic effects and by increased expression of tumour-derived interleukin IL-8. IL-8 expression has been found elevated in human RCC tumours with intrinsic resistance to Sunitinib, indicating that IL-8 levels may be useful as a predictive biomarker for clinical response to the drug. Moreover, in RCC, the extracellular matrix metalloproteinase inducer

(EMMPRIN) was also reported as associated with Sunitinib resistance [201]. RCC studies on how cells adapt to VEGF-targeted antiangiogenesis *in vitro* revealed that the resistance could be the result of VEGF-independent angiogenesis pathways activation. Moreover, also in RCC, Sunitinib resistance has been associated with intratumoural drug sequestration in lysozymes. Further studies are required to confirm these hypotheses in order to understand how it will be possible to overcome Sunitinib resistance and offer more innovative therapeutic strategies/approaches that could be translated to a better patients clinical outcome [186].

1.7 EVEROLIMUS AND PIK3/AKT/mTOR PATHWAY IN NEUROENDOCRINE NEOPLASMS

Since NENs management has demonstrated to be challenging, effective and safe therapeutic options have always been actively pursued in clinical research. Several pathways were studied in order to find new perspectives and the PI3K/AKT/mTOR pathway, frequently over activated in cancer, was individuated as good candidate for the development of new therapeutic strategies. A graphical illustration of the pathway is represented in figure 3 [202].

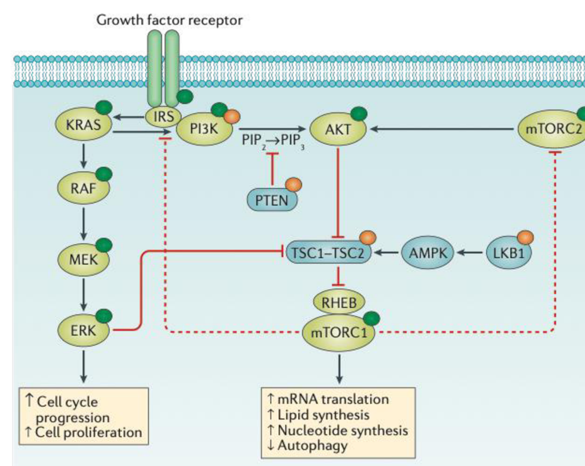

Figure 3: *intracellular signalling via the PI3K/AKT/mTOR pathway* [202].

This pathway plays a crucial role in cell growth and survival and, therefore, is a frequent target

for pharmacologic intervention [196, 203]. The first PI3K pathway-targeted agents approved for the treatment of cancer were the rapamycin analogues ("rapalogs") Everolimus and Temsirolimus, which allosterically inhibit one of the mTOR subunits. Ranges of investigational agents, which have as main target other components of the PI3K pathway, have followed these inhibitors. These inhibitors include ATP-competitive dual inhibitors of class I PI3K and mTOR, "pan- PI3K" inhibitors (which inhibit all 4 isoforms of class I PI3K), isoform-specific inhibitors of the various PI3K isoforms, inhibitors of AKT and ATP-competitive inhibitors of mTOR only [204]. Since a bulk of preclinical evidences have shown that the PI3K/AKT/mTOR signalling pathway plays a central role in the pathogenesis and progression of NENs, rapalogs started to be studied also in NENs. Everolimus was found to be a well-grounded strategy for the treatment of NENs and its efficacy and safety were widely demonstrated in the RADIANT trials that have lead to its approval for pNENs in 2011 and GIST-NENs/BP-NENs in 2017 [130].

1.7.1 PIK3/AKT/mTOR PATHWAY

PI3K/AKT/mTOR signalling pathway has been shown to be deregulated in several human malignancies, including NENs. Deregulated mechanisms involve overexpression or activation of RTKs, mutations in PI3K, mutations/ amplifications of AKT, TSC2 inhibition and others. mTOR is a serine/threonine kinase intermediary within PI3K/AKT pathway that recognizes stress signals (nutrient/energy depletion, oxidative or hypoxic stress and proliferative and survival signals) and regulates cell survival, proliferation and apoptosis [205]. mTOR acts as the catalytic subunit of two large complexes, mTORC1 and mTORC2. These complexes reflect distinct roles in cell functions regulation and have different upstream and downstream interactions. mTORC1 serves as an integrate sensor of energy status and nutrients, growth factors, oxygen and stress. mTORC1 balance, in response to environmental conditions, cells anabolism and catabolism. Its activation

status regulates important cell processes such as protein/lipid synthesis and autophagy. A key component of mTORC1 is Raptor: the regulatory-associated protein of mammalian target of rapamycin. On the other hand, mTORC2 complex includes mTOR, rictor (rapamycin-insensitive companion of mTOR), mLST8, mSin1 (also known as mitogen-activated-protein-kinase-associated protein 1), Protor, Hsp70 and DEPTOR. mTORC2 has a less defined role in comparison with mTORC1 and is considered a well distinct branch of the PI3K/Akt/mTOR pathway. mTORC1 and 2 show different sensitivity to rapamycin: mTORC1 is rapidly inhibited by rapamycin, while mTORC2 inhibition needs longer exposure [(203, 206)]. TSC2 is a negative mTORC1 regulator and its activity is itself regulated by PI3K/AKT signalling. Once activated, mTORC1, though a series of phosphorylation, triggers its effectors, 4EBP1(eIF4E Binding Protein) and S6K1 (p70S6 Kinase 1). These two proteins enhance cell proliferation, survival and angiogenesis through the regulation of Cyclin D1, Bcl-2, Bcl-xL, hypoxia-inducible factor-1 (HIF-1) and VEGF. This complex is less understood than mTORC1 but recent works have demonstrated that it directly phosphorylates AKT, PKC-α, and paxillin (focal adhesion- associated adaptor protein), regulates the activity of the small GTPases Rac and Rho that are related to cell survival, migration and regulation of actin cytoskeleton [(207, 208)]. AKT phosphorylation remains the most important function of mTORC2 and, once active, AKT promotes cell survival and proliferation, through the phosphorylation/inhibition of several key substrates including the FoxO1/3a transcription factors, the metabolic regulator GSK3β and the mTORC1 inhibitor TSC2. Finally, mTORC2 also phosphorylates and activates SGK1, another AGC-kinase that regulates ion transport as well as cell survival. The mTORC1 signalling cascade is activated by phosphorylated AKT, which in turn, for its activation, requests the activation of PI3K by RTKs activation. These receptors interact with the p85 regulatory subunit of PI3K. p85 dimerizes with the p110 catalytic subunit of PI3K and localizes the p85/p110 heterodimer to the plasma membrane [(209, 210)]. Upon activation, the p110 subunit of PI3K phosphorylates lipids on the plasma membrane promoting the conversion of PIP2

(phosphatidylinositol-4-5-bisphospate) in PIP3, which recruits AKT to the plasma membrane. Interaction with PIP3 causes conformational changes in AKT that expose its phosphorylation site Thr308 in the kinase domain and Ser473 in the C-terminal domain. AKT, to be activated, needs two phosphorylation simultaneously: one on Thr308 and one Ser473 residues, respectively induced by PDK1 (activated by PI3K) and mTORC2. Moreover, RTKs activation lead to inhibition of TSC complex and, therefore, to mTORC1 activation [(211)].

Given the key role of mTOR in cell growth and metabolism, it is predictable the existence of an association between mTOR pathway activity and pathological states, including cancer. Deregulation of PI3K/AKT/mTOR signalling pathway is one of the most common mechanisms of tumorigenesis and, alterations of this pathway, have been reported several times in many types of cancer including NENs [(212, 213)]. In general, the two widest discovered mechanisms of PI3K/AKT activation in human cancers are triggered by RTKs and somatic mutations in specific elements of its signalling pathway. The most well known aberrant alterations involve mutations of the p110α isoform of the PI3K sequence, loss of function of the tumour suppresser gene PTEN and abnormalities of AKT and RAS [(214)]. The major PI3K gene alteration is described to occur in the p110α coding region and consist in its amplification; this has been documented in several malignancies such as ovarian and cervix cancer. PTEN is a protein that dephosphorylates PIP3 and, in this way, acts as a negative regulator for PI3K- induced signalling. PTEN is often mutated in the advanced stages of various human tumours, including prostate, glioblastoma, melanoma and endometrial carcinoma. Deletions of 10q, including PTEN region, are found in 24%-58% of invasive urothelial carcinomas whereas PTEN-inactivating mutations have been found in approximately 30% of primary glioblastoma. A somatic mutation in AKT1 was discovered in 6% of colorectal cancers, 2% of ovarian cancers and 8% of breast cancers [(215, 216)]. Concerning PI3K/AKT/mTOR pathway alterations in NENs, several abnormalities have been reported during the latest years. What appears clear in NENs is that PI3K/Akt/mTOR pathway aberrant activations

are driven by a number of deregulated RTKs activities that lead to an over activation of their downstream effectors [217]. Analysis conducted in pNENs mouse xenograft models revealed that PI3K/AKT pathway might promote metastasis and invasion in these tumour cells: knockdown of AKT1, AKT2, or AKT3 impairs neuroendocrine cell invasion whether knockdown of PTEN promotes liver metastasis. Moreover, pNENs gene expression profiling demonstrated that PTEN and TSC2 are down regulated in 50 and 35% of patients, respectively, and down regulation is predictive of poor survival. Immunohistochemistry is also suggestive of deregulation of the PI3K/AKT pathway, indeed analysis have revealed that PTEN expression is reduced or altered in patients tissues samples. Assessment of pAKT expression in GEP-NENs suggests that the PI3K/AKT pathway is plentifully active in these tumours. Immunohistochemistry analysis demonstrated a high percentage of pAKT(Ser473) in a group of NENs mixed sample from various sites and a significant association between pAKT(Ser473) and pEGFR expression and elevated Ki67 [218, 219, 220]. Furthermore, genetic (exomic) sequencing studies of non-familial pNENs have revealed that mutations in the PI3K/AKT/mTOR pathway occur in 15% of pNENs and mTOR overexpression in well-differentiated pNENs have been frequently reported. Additionally, miR-21 overexpression has been strongly associated with both a high Ki-67 proliferation index and liver metastasis presence. miRNA expression profiling showed that the genetic regulator miR-21 and Ki-67 index are inversely proportional to PTEN levels [213].

1.7.2 EVEROLIMUS AND NEUROENDOCRINE NEOPLASMS

Rapamycin was isolated in 1975 as an antibiotic product of the actinomycete Streptomyces hygroscopicus and was initially investigated as antifungal agent. In 1977 its immunosuppressive activity was described for the first time and, since this first observation, much has been learned about the complex mechanisms of action of this macrolide. mTOR inhibition by rapalogs is

exerted by the formation of an intracellular complex between rapamycin and intracellular immunophilin FK506 binding protein 1 A 12 kDa (FKBP12) [(221)]. This complex inhibits the kinase activity of mTOR by restricting active site access and directly blocking substrate recruitment. While rapalogs almost completely inhibit mTORC1, mTORC2 is affected only after long exposure to the molecule. Inhibition of mTORC1 is an effective anti-tumoral strategy in several cancers including NENs. The first mTOR inhibitor introduced in clinic was Temsirolimus. This drug showed potent immunosuppressive and antiproliferative properties and has been approved by FDA and EMA for advanced RCC and mantle cell lymphoma. In NENs, a Phase II study in 37 advanced tumours showed modest efficacy of the drug [(202)]. The most well known mTORC1 inhibitors is Everolimus that is currently approved for treatment of pNENs, gastrointestinal NENs and lung NENs. Everolimus' NENs approval story started with RADIANT-1, a multicentre Phase II trial, in which this drug was tested alone or in combination with monthly Octreotide LAR, in 160 patients with advanced pNEN. Results indicated that the two drugs combination controlled tumour growth and that in patients receiving Everolimus alone median PFS was 9.7 months and median OS was 24.9 months. These encouraging results were exploited in a prospective randomized Phase III trial (RADIANT-2). In this study, Everolimus in combination with Octreotide LAR were compared with placebo in advanced NENs with carcinoid syndrome. Results showed reduction of disease progression with an increased PFS, 16.4 months versus 11.3 months, in favour of patients treated with Everolimus plus Octreotide LAR versus placebo arm. This trial included patients with NENs originated in different organs (as small intestine, lung, pancreas and others) and results were subjected to high biological and/or clinical heterogeneity, possibly preventing conclusive results on Everolimus efficacy [(205, 130, 222)]. Thus, a larger Phase III study (RADIANT-3) was realised in a cohort of 410 patients with pNENs randomly assigned to Everolimus, n = 207, or placebo, n = 203. Significant prolonged PFS in patients treated with Everolimus (11.0 months versus 4.6 months) was observed and have led to drug's approval for pNENs in 2011 [(223)]. Another trial, RADIANT-4, has

been completed in recent years leading to the approval of Everolimus for patient with gastrointestinal and lung NENs. The study has indeed demonstrated prolonged PFS in patients treated with Everolimus compared with those treated with placebo (11.0 months versus 3.9 months) [224]. Other PI3K/AKT/mTOR pathway inhibitors have been evaluated in NENs such as PI3K inhibitors, AKT inhibitors and mTORC1/mTORC2 inhibitors but no one has been approved for the treatment on these malignancies. Also several strategies were evaluated such as combination between mTORC1 and EGF inhibitors or mTORC1 and SSAs. However, no significant results were obtained [196].

1.7.3 RESISTANCE TO EVEROLIMUS

Despite the encouraging results obtained during trials, Everolimus showed a high initial antitumor activity followed, after a first period of treatment, by disease progression. Moreover, patients can be not sensitive to the drug from the beginning. These mechanisms involve primary and acquired resistance that can limit the efficacy of targeted therapies in NENs [181]. Understanding and overcoming resistance may be important in preventing treatment discontinuation due to disease progression leading to an ad hoc treatment for each patient. Several hypotheses have been postulated regarding the mechanisms involved in Everolimus resistance. The most well known mechanism is due to the compensatory up regulation of PI3K/AKT cascade after treatment with rapamycin and its analogues. This compensatory effect leads to an increase of pAKT T308 and pAKT S473 and, as a consequence, to treatment resistance. In other words, upstream AKT phosphorylation is promoted by Everolimus inability to block mTORC2 [225, 226, 227]. The inhibition of the S6K negative feedback via IGF1/IGF1R signalling is another postulated mechanism of resistance. Insulin receptor substrate-1 (IRS-1) is normally phosphorylated by p70S6K and, therefore, is under basal negative regulation. mTOR inhibition prevents IRS-1 phosphorylation allowing IRS-1 to complex with IGF1R and, as a result, promotes AKT signalling [228, 229].

Moreover, mTOR resistance mechanisms include: activation of alternative pathways (such as ERK pathway), mutation in rapalog targets FKBP- 12 or mTOR, loss of function of PP2A (a phosphatase involved in dephosphorylation and inactivation of AKT) and autophagy stimulation [230]. Constitutive PI3K activation can also occur thanks to downregulation and/or mutational loss of function of PTEN, which leads to unregulated AKT activation. Indeed, the upstream mTOR regulators, PTEN and TSC2, have been found often mutated/downregulated/altered in their protein expression level, causing mTOR activation in pNENs. Moreover, PTEN loss was evidenced in 63% of small cell neuroendocrine carcinomas (17/27) and an immunohistochemical (IHC) analysis in GEP-NENs showed high expression and activity of mTOR, 4EBP1, cytoplasmic phospho-4EBP1 (p4EBP1), nuclear p4EBP1, phospho-S6K (pS6K) and phospho-eIF4E (peIF4E). These alterations have been correlated with a shorter PFS in most of the cases and with an enhanced tumour proliferative capacity. During these studies, observed differences in mTOR activity and expression levels were also inversely related with the variation in rapalogs response. This hypothesis was also sustained by other findings in which a high expression of mTOR and its downstream target RPS6KB1 were related with an adverse clinical outcome in NENs [179, 205, 231].

To avoid or delay development of acquired resistance, double inhibition of targets by drugs combination has been investigated. Emerging evidences show that dual inhibition of these pathways, particularly PI3K/AKT and mTOR pathways, could be a novel therapeutic approach to overcome Everolimus resistance. However, for the moment, no combined therapies have been approved for NENs treatment [203].

1.8 NEW FRONTIERES IN THE TREATMENT OF NEUROENDOCRINE NEOPLASMS

NENs represent a clinical challenge due to heterogeneity of their biological behaviour, diagnosis and treatment options. PRRTs and targeted agents have changed the management of NENs

and, in latest years, the idea of a multidisciplinary approach has became considered necessary for an optimal patients management. Several new approaches are currently studied for the individuation of an effective therapy in NENs [132]. Indeed, recent studies on Bevacizumab, monoclonal antibody directed against VEGF, demonstrated that it could be incorporated into future trials of advanced GEP-NENs. A randomized phase III trial compared Octreotide plus IFNα-2β or Bevacizumab in advanced GEP- NENs while a phase II study investigated Bevacizumab and Temozolomide combination in advanced pNENs demonstrating, in both cases, a good partial response [232]. Moreover, immunotherapy has been investigated in a number of malignancies exhibiting substantial results in patients with melanomas and some tumours with neuroendocrine origin. Unfortunately, up to now, only few data are available in NENs and therefore more studies are needed to introduce these compounds in medical treatment [233]. Furthermore, other RTKs inhibitors have been evaluated for NENs treatment. A plethora of new or emerging RTKs inhibitors are approved for use in other solid tumours and are awaiting clinical trials to validate their application in NENs. The main ones are: Pazopanib, that has shown to be effective in advanced pNENs, Axitinib, that has proven to have antitumoral effects in advanced extrapancreatic NENs and Sorafenib, that has shown to be effective in combination with Bevacizumab in advanced NENs. Moreover, more recent RTKs inhibitors include Cabozantinib, Lenvatinib, Sulfatinib and Famitinib that are awaiting for results of ongoing trials [234]. Besides molecular targeted therapies, there are also other agents that have been recently approved for NENs treatment as Lutathera ([^{177}Lu]Lu-DOTA-TATE). This is a radiopharmaceutical for PPRT that have been approved by the EMA in 2017 and the FDA in 2018 for the treatment of SSTR positive GEP-NENs. Its approval followed the multicentre phase-III clinical trial NETTER-1 that compared the treatment with Lutathera with the treatment with high doses of Octreotide LAR [235]. Moreover, another recently approved agent for the treatment of some NENs, is Telotristat ethyl. This agent is a serotonin synthesis inhibitor that can be used as a therapeutic option in patients with CS diarrhoea [236]. The evolution of therapeutic modalities of

NENs is illustrated in figure 4 (10).

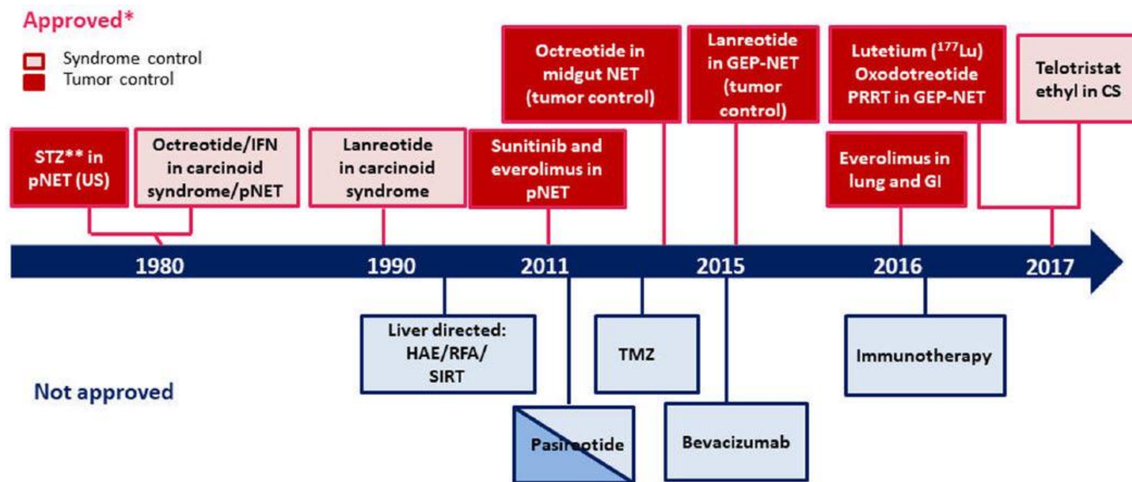

Figure 4: *evolution of NENs therapeutic modalities* (10).

As illustrated, several new NENs treatment frontiers are studied from research community. What is desirable is the identification of specific molecular alterations that might expand currently available therapeutic options allowing a personalised approach.

1.8.1 DINACICLIB AND THE INHIBITION OF CELL CYCLE PROGRESSION

Cell cycle is a cellular event composed by a series of steps in which cellular components are doubled, and then accurately segregated into daughter cells. In eukaryotes, DNA replication happens in the S-phase (synthesis phase), and chromosome segregation occurs during Mitosis or M-phase. Two Gap phases separate S phase and mitosis and are known as G1 and G2 phases. During G1 and G2, cells prepare all the necessary components for replication and, therefore, activate a series of processes in order to gain mass, integrate growth signals and organize a replicated genome (237). Cell cycle progression is regulated by extracellular signals from the environment, as well as by internal signals that monitor and coordinate the various processes that take place during different cell cycle phases. These phases are coordinated by cyclin-dependent kinases (CDKs), cyclin-

dependent kinase inhibitors (CKIs) and cyclins. These proteins stimulate and inhibit each other exerting cell cycle control and subsequent transcriptional regulation. CDKs are serine/threonine protein kinases that phosphorylate key substrates to promote DNA synthesis and mitotic progression [(238)]. CDKs are activated by their binding with cyclins that are proteins tightly regulated at both the levels of synthesis and ubiquitin-dependent proteolysis. CDK/cyclins drive cell cycle progression: progression from G_1 to S phase is mainly regulated by Cdk2 and Cdk4 (and in some cells CDK6) in association with cyclins D and E while CDK4 and CDK6 with the D-type cyclins (cyclin D1, D2, and D3) play a critical role in progression through G_1. Moreover, CDK2/cyclin E complexes are required for the G_1 to S transition and initiation of DNA synthesis while CDK2 with cyclin A act in the progression of cells through S phase [(239)]. CDKs activity is negatively regulated by the binding of small inhibitory proteins, the CKIs, or by inhibitory tyrosine phosphorylation, which blocks phosphate transfer to substrates. CDK/cyclin complexes formation is regulated by two families of CKIs: Cip/Kip family and Ink4 family. Cip/Kip family regulates all stages of progression through G_1 and S phase by inhibiting the formation of the complexes between of CDK2, 4, and 6 with cyclins A, D, and E. In contrast, members of the Ink4 family, are specific for complexes of Cdk4 and 6 with cyclin D [(240)]. Therefore, this delicate process, that has been reported as altered in several malignancies, regulates cell cycle progression. Several studies have identified deregulated CDKs and CKIs as main effectors of uncontrolled cell proliferation and, therefore, over the past 20 years, a number of CDK inhibitors have been developed and successfully tested in clinical trials for different tumour types [(241)]. Numerous CDK inhibitors, including Dinaciclib (Merck, Kenilworth, NJ), Palbociclib (Pfizer, New York, NY), Abemaciclib (Lilly, Southlake, TX), BAY1000394 (Bayer Healthcare, Leverkusen, Germany) and Ribociclib (Novartis Pharmaceuticals Corp., Basel, Switzerland) are currently being tested in clinical trials for various advanced cancers. Palbociclib demonstrated to have anticancer efficacy in both preclinical studies and in a subset of patients in clinical trials with estrogen receptor–positive breast cancer. This agent appeared to be

particularly effective in combination with the aromatase inhibitor Letrozole and has been approved from FDA in 2015 for the treatment of estrogen receptor-positive, HER2-negative breast cancer [242]. Dinaciclib is a potent CDK1, -2, -5, -9 inhibitor that has been proved to induce apoptosis in different tumour cells. Dinaciclib exerts its action at different levels: cell cycle regulation (through CDK1, -2 inhibition), actin polymerization control (through CDK5 inhibition) and RNA-polymerase II regulation (through CDK9 inhibition). Despite Dinaciclib has shown to induce solid tumours regression in mouse models and cell-cycle progression inhibition in multiple tumour cell lines, reports of randomized phase 2 trials in solid tumours have been disappointing, showing no significant response in patients with non-small cell lung cancer or acute lymphoblastic leukaemia [243, 244]. Anti-cancer activity was found to be encouraging, but not sufficient for planning monotherapy treatments. However, due to these positive preliminary results, future studies might lead to new awareness and potential Dinaciclib applications [245].

1.8.2 LINSITINIB, ERLOTINIB AND THE INHIBITION OF SPECIFIC TYROSINE KINASE RECEPTORS

As illustrated before, RTKs play an essential role as oncogenes in NENs and the approved agent Sunitinib has shown some limitations as therapeutic agent. Several other drugs, with a more selective target, have been tested in latest years and *in vitro* and *in vivo* studies have demonstrated activity of different RTKs inhibitors in neuroendocrine cells and tumours [234]. Erlotinib is a potent first-generation inhibitor of EGFR, and is an established first-line therapy for patients with NSCLC positive for exon 19 deletions or exon 21 mutations. Erlotinib combined with Gemcitabine has also been approved for systemic treatment in advanced, non-operable pancreatic carcinoma. This drug blocks MAPK and PI3K-AKT pathways suppressing the signalling though EGFR inhibition [246]. Benefits of Erlotinib are mainly based on tumour control and OS improvement rather than

complete remission rates and rapid tumour responses. Erlotinib has been examined in phase I and II studies for several malignancies including hepatocellular carcinoma, colorectal, biliary, gastric, breast, ovarian, endometrial and renal cell cancer. However, in these cases, drug efficacy in terms of OS and response rates was low. In contrast, Erlotinib combination with other drugs has shown to be promising in recurrent or metastatic squamous cell cancer of the head and neck and other malignancies (247). Linsitinib (OSI-906) is a powerful, orally bioavailable dual IGF1R and IR tyrosine kinase inhibitor that has shown antiproliferative effects in a variety of tumour cell lines, and antitumor activity in an IGF1R-driven xenograft model. Phase II clinical trials were initiated for several malignancies such as multiple myeloma, ovarian cancer, hepatocellular carcinoma and NSCLC while phase III clinical trial was started for adrenocortical carcinoma. However, non-completely satisfactory results have led to studies discontinuation. During trials, Linsitinib has shown a good action once in combination with EGFR inhibitors suppressing resistance to single receptor blockade. Preclinical data have indeed pointed out that IGF1R mediates Erlotinib acquired resistance in lung cancers with wild-type EGFR, and combined inhibition of IGF1R/IR and EGFR can have a supra-additive inhibition of tumour growth *in vitro* and *in vivo* in NSCLC, breast, pancreatic, and colorectal cancers (248, 249).

1.9 NEW APPROACHES IN THE STUDY OF NEUROENDOCRINE NEOPLASMS MEDICAL THERAPY

As described above, NENs therapy includes several approaches such as cytotoxic agents, SSAs, PPRT and targeted agents. Despite comprehensive and interesting medical progresses, the current available therapeutic options are still inadequate for NENs treatment, mainly due to the lack of in-depth knowledge of molecular resistance mechanisms (197). Novel strategies are therefore needed especially for refractory and/or recurrent NENs with a poor prognosis. The term "novel

strategies" can be referred both to novel agents and targets but also to novel techniques that can be applied to better understand the mechanisms of already approved therapies. New strategies should therefore investigate molecular mechanisms responsible for NENs heterogeneity, tumour interactions with adjacent tissues and the mechanisms behind the different responses to treatments (250). Increase our knowledge and guarantee the development of new promising therapies for NENs treatment is therefore mandatory.

1.9.1 3D CELL CULTURE METHODS

The development of several therapeutic agents leads to their imperative *in vitro* assessment before clinical trials start. Tests can be performed using different model systems and the two most important systems used today for drug testing are conventional 2D systems (monolayers) and 3D tumour cultures. In research against cancer the choice of the most appropriate cell culture method may lead to a better comprehension of tumour biology and, therefore, optimize already approved therapies, and find new treatment strategies. Therefore, the choice represents then a crucial point, which should not be underestimated (251). 2D systems have shown during years several limitations mostly due to the unnatural cells disposition; these limitations can partially be overcome using 3D cultures that are indeed garnering huge attention from scientific community. Since 3D cultures can partially recapitulate tumour structure and microenvironment, 3D cell cultures have been employed in the attempt to fill the gap between *in vitro* and *in vivo* systems (252). 3D cultures have shown to have different benefits but the most important is that this approach provides a more accurate representation of a solid tumour mass. This fact leads to the generation of different proliferation areas and, therefore, to different gene expression patterns and cellular behaviours in the spheroid that cannot be replicated through 2D systems (253, 254). Other additional 3D cultures features involve enhanced cells interaction and crosstalk. These connections contribute to the generation of a

complex microenvironment that, again, cannot be reproduced in 2D cultures and may represent an essential aspect for drug discovery research [255]. In order to bridge the gap between monolayers and expansive models, such as animal models, many 3D culture techniques have been optimized during the last 40 years and are available for scientific research [256]. These methods involve different strategies for cell aggregation and, therefore, different tools and methodologies that can be exploited in different ways depending on the purpose of the study. Independently of the 3D method take in consideration, 3D cell cultures appear to be less sensitive to drugs than 2D cultures. This observation is not a surprise since 3D structures generate less access to compounds in the medium and can contribute to change cells response due to hypoxia condition and cell cycle alterations. Different responses in drug-testing can also be observed between different 3D culture methods. These methods can be mainly divided into two groups according to the presence/absence of a scaffold [257]. Scaffolds are usually made with biodegradable materials such as silk, collagen, laminin and alginate. The most relevant problem related to 3D cultures with scaffolds is that materials may affect cell behaviour and growth and, as a consequence, void experiments output. On the other hand, scaffold-free allow to generate 3D cell cultures in a simple and fast way that, however, may involve the use of expansive plates. Nevertheless, the latter method appears to be the best in recreating interaction between cells without their alteration [258, 259]. 3D cell cultures obtained with different techniques have also been used in recent years to study NENs and are entering day by day as a common lab practice [260, 261, 262]. 2D culture system is still the most common research model. However, its limitations cannot be denied and 2D cultures are increasingly been seen as an inefficient model [251]. The 3D models are potentially a better approach in the search for new biomarkers and new treatment strategies, leading us closer to what we have previously defined NENs research goal: a personalized approach.

1.9.2 TUMOUR MICROENVIRONMENT AND CANCER

Tumour microenvironment (TME) can be defined as a complex entity composed by multiple cell types that variably interact by heterotypic cross talk within themselves and with the surrounding cell structures. The immune cells, capillaries, basement membrane, activated fibroblasts and extracellular matrix (ECM) surrounding the cancer cells constitute the TME. In the past, malignancies were thought to be as separate masses of proliferating cancer cells but now the concept has evolved leading to the new awareness of a complex system. This system is deeply involved in tumour cell growth, behaviour and cancer cells response to medical therapy. What has been found in several malignancies is the instauration of complex interactions between every component of TME that result in a unique entity with the same aim: grow, proliferate, and invade. Tumour cells promote a stromal metabolic reprogramming with the generation of a “feedback loop” in which microenvironmental cells itself drives metabolic changes in cancer cells and/or provide metabolic resources required for tumour growth [(263)]. Every component contributes to cancer cells epigenetic deregulations, acquisition of invasive and metastatic capabilities, as well as induction of both local and systemic immune suppression. More than one evidence has been provided regarding the involvement of TME in NENs resistance/tumour progression and what is more important is that, at the moment, system vulnerabilities are not therapeutically exploited in molecular therapy [(264)]. TME importance in NENs development has been underlined from one of the most widely used models to study these malignancies: the RIP1-Tag2 mouse. In this setting, tumours arise from stochastic events in single cells, go through distinct development stages and grow in their correct environment. This process leads to a complex entity that recapitulates disease key characteristics. The predictive value of this model has been confirmed during Everolimus and Sunitinib studies that have shown quite consistent parallelism between patients PFS and mouse OS when treated with these agents [(148)]. For that reason, in this context, the dynamic cross talk between NENs cells and reactive stroma appear to be crucial in regulation of tumour growth and progression. Several

components generate the TME such as the ECM, stromal cells, endothelial cells and immune cells. A summary of TME components is represented in figure 5 [268].

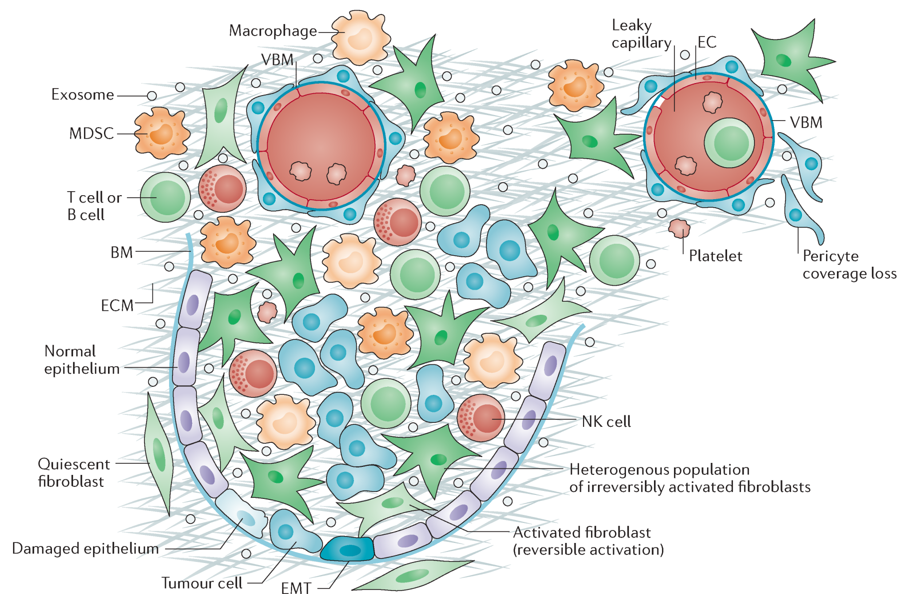

Figure 5: *the heterogeneous population of TME* [268].

- ECM: An array of multidomain macromolecules organized in a cell/tissue-specific manner generate the ECM intricate network. ECM Components are linked together forming a structurally stable composite, contributing to the mechanical properties of tissues. Growth factors and bioactive molecules are preserved in ECM that, in this sense, acts as a reservoir of different factors. Proliferation, adhesion, migration, polarity, differentiation, and apoptosis of cancer cells are controlled by this highly dynamic entity that assume a vital importance for tumour development [265]. The specific composition of the matrix controls tissue biochemical and biomechanical properties in a very strict way, and peculiar ECM alterations have been described in NENs. Several differences between normal ECM and cancer ECM have been observed in particular for GEP-NENs. In pNENs fibulin-3, periostin, fibrinogens, and kininogens were found to be significantly up regulated whereas decorin, hemicentin-1, lectin, galectin-1, and mannose binding 1 were shown to be down

regulated [266]. On the other hand, not much is known regarding ECM composition in pulmonary NENs. One of the few evidences have been demonstrated in an histochemistry study of 55 typical lung NENs in which a higher density of collagen and elastic fibres have shown to be associated with increased tumour size and nodal metastases [267]. Moreover, ECM degradation, promoted from different matrix-metalloproteinases often up regulated in cancer, was shown to contribute to NENs development, progression, and aggressiveness through different mechanisms. These mechanisms involve chemoattractants generation (useful for inflammatory cells recruitment), release/activation of proangiogenic factors and changes in ECM elasticity, connected to cells behaviour changes though a mechanosensitive pathway [264].

- Stromal cells: the acronym CAFs indicates cancer-associated fibroblasts that, in the past decade, have cemented themselves as key components of tumour progression. Recent findings suggest that they probably contribute to a wide range of fibrotic stromal programmes of many different tumours [268]. Increasing evidence indicates that a cross talk exists between fibroblasts and NENs cells, as well as between fibroblasts and endothelial cells or inflammatory cells. NENs cells enhance CAFs activation leading to a huge production of growth factors, chemokines, ECM and, moreover, an increase of endothelial cells and pericytes angiogenic recruitment. The most well known factors secreted by NENs able to stimulate CAFs activities are serotonin, TGF-β, and PDGF; these factors are deeply important for cancer progression and have been documented as crucial in CAFs activation from several studies [264, 269]. While the effects of NENs cells on the reactive stroma have been partially characterized, the modifications induced by CAFs to tumour cells still remain poorly understood. In this context Bowden *et al.*, through a proteomic approach, have recently screened *in vitro* a panel of 55 proteins with known carcinogenic associations and potentially secreted by CAFs in NENs. This screening revealed that IL-6, VEGF, and

monocyte chemoattractant protein 1 are actively secreted by CAFs, indicating that these cytokines can act as inducers of NENs cell proliferation [(270)].

- Endothelial cells: angiogenesis is the process leading to the formation of new blood vessels and is an essential process for tumour progression. Oxygen and nutrients are provided to the tumour by blood vessels that are also needed for enhance tumour growth, remove waste products from tumour tissues and provide a gateway for tumour metastasis [(271)]. Blood vessels consist of tumour endothelial cells (TECs), situated in the inside of the blood vessels and perivascular cells, which surround the blood vessels externally. Endothelial cells (ECs) are almost quiescent and became activated only in case of stimulation, usually generated by an insult, promoted by VEGF. Tumour angiogenesis is one of the most important processes in cancer progression since it provides a channel for metastasis occurrence [(272)]. NENs are among the most extensively vascularized cancers and this is not a surprise since, physiologic functions of normal endocrine tissue, require a high vascular supplement. NENs intratumoral vessel density has been approximately estimated to be 10-fold higher as compared with carcinomas. In contrast with other neoplasms, in pNENs has been observed that a high intratumoral microvascular density is associated with good prognosis and prolonged survival. This fact is indeed called the "neuroendocrine paradox" and its biological significance has still to be elucidated. On the other hand, what is well known is that NENs cells overexpress a plethora of proangiogenic factors including VEGF, FGF, PDGF, semaphorins and angiopoietins as a result of HIF1α aberrant hyperactivation [(269, 273)]. Up to 80% of GEP- NENs overexpressed VEGF and its overexpression is higher in well-differentiated neoplasms rather than poorly differentiated NENs. In contrast with pNENs, in pulmonary NENs the role of VEGF has been poorly studied and the few available results are in contrast to each other in terms of correlation between expression of this growth factor, microvascular density, and patient prognosis. Other key factors for NENs progression that

have been recently described are angiopoietins. Indeed, angiopoietin-2 has been found significantly up regulated in pNENs, and the ability of this molecule in increasing the microvascular density of pNENs xenografts has been demonstrated by in vivo evidences [264].

- Immune cells: Tumour progression is also influenced by a huge variety of immune cells. Indeed, malignancies are characterized by a high heterogeneity of immune cells population that is not only variable between tumour types, but also within one patient or between different patients with the same cancer type [274]. NENs immune contexture has become clearer in recent years and several immune cells have been reported to infiltrate NENs. These immune cells include B and T cells, NK cells, mast cells, dendritic cells and macrophages; this complex system generate an immunosuppressed microenvironment able to enhance tumour progression. Data indicates that T cells infiltration was detectable in 68% of samples in a series of 87 pNENs and this fact was not associated with tumour grade. Moreover, CD8+ lymphocytes were found as infiltrating cells in both low- and high-grade pulmonary NENs and this has been reported as an independent predictor of improved OS and PFS [275]. Another kind of immune cells, NK cells, has also demonstrated to impair cytolytic activity in GEP-NENs patients and their activity has been related to disease status. Dendritic cell activity has also been described in NENs and studies have reported systemic derangements of this cell type. In particular, an altered expression of MHC class I molecules has been demonstrated in 10/11 samples of pNENs [264, 269]. Tumours immune response has been extensively described and analysed for many years and have lead to the development of immunotherapy. Immune cells, therefore, can represent an important target that can be exploited for the generation of immunotherapies. There are currently several clinical trials testing antibodies in different stages of GEP-NENs (mostly high-grade tumours). However, latest evidences suggest that only a minority of these malignancies may be effectively

targeted with immune checkpoint inhibitors [275].

These new insights on TME have led and will lead to new prospective in cancer therapy and treatment resistance. Indeed, what appears clear is that targeting stromal cells metabolism can influence tumour progression to the same extent as targeting the tumour cells metabolic mediators. Therefore, target only cancer cells in medical treatments can be insufficient. Development of therapeutic medical approaches must, therefore, take into account that the metabolic reprogramming of tumour cells is flexible and evolves along with microenvironmental changes [263]. Moreover, cytokines in the microenvironment not only can contribute to tumour growth, invasion, and metastasis, but in some situations can also drive the development of drug resistance. Since resistance is a major cause of treatment failure in cancer, this issue must be taken in consideration [276].

In this prospective, the elaboration of combined therapies able to target both TME and cancer cells seems to be imperative. However, before stopping the microenvironment components, it should be desirable to understand which mechanisms undergo between these complex system elements.

1.10 PREVIOUS FINDINGS

In 2013 or research group published a BP-NENs study which aims was to assess the effects of a novel dual PI3K/mTOR inhibitor on BP-NENs cell lines in comparison with Everolimus. Gagliano *et al.* verified NCI-H720 and NCI-H727 cell viability after treatment with Everolimus at different concentrations for 72h (figure 6A) [277]. This study identified 100 nM Everolimus as the most useful concentration to perform cell viability studies in these cell lines. As a consequence, this concentration has been used since then, also on the basis of studies from other research groups [278, 279]. Figure 6A also shows the effect of NVP-BEZ235 on NCI-H720 and NCI-H727 cell viability: this compound exerts a higher anti-proliferative effect on both cell lines, but it is not capable to

overcome Everolimus resistance [277]. Therefore, NVP-BEZ235 was not employed in further experiments. Concerning Dinaciclib tests, NCI-H720 and NCI-H727 cell viability results obtained from treatments with the drug for 96h are shown in figure 6B. Dinaciclib has shown promising antitumor activity in preclinical studies in a broad spectrum of cell lines with median IC50 of 11 nM. However, on the basis of the results shown in figure 6B and due to the need to perform several experiments on 3D culture system, the chosen concentration in this study has been 50 nM [280].

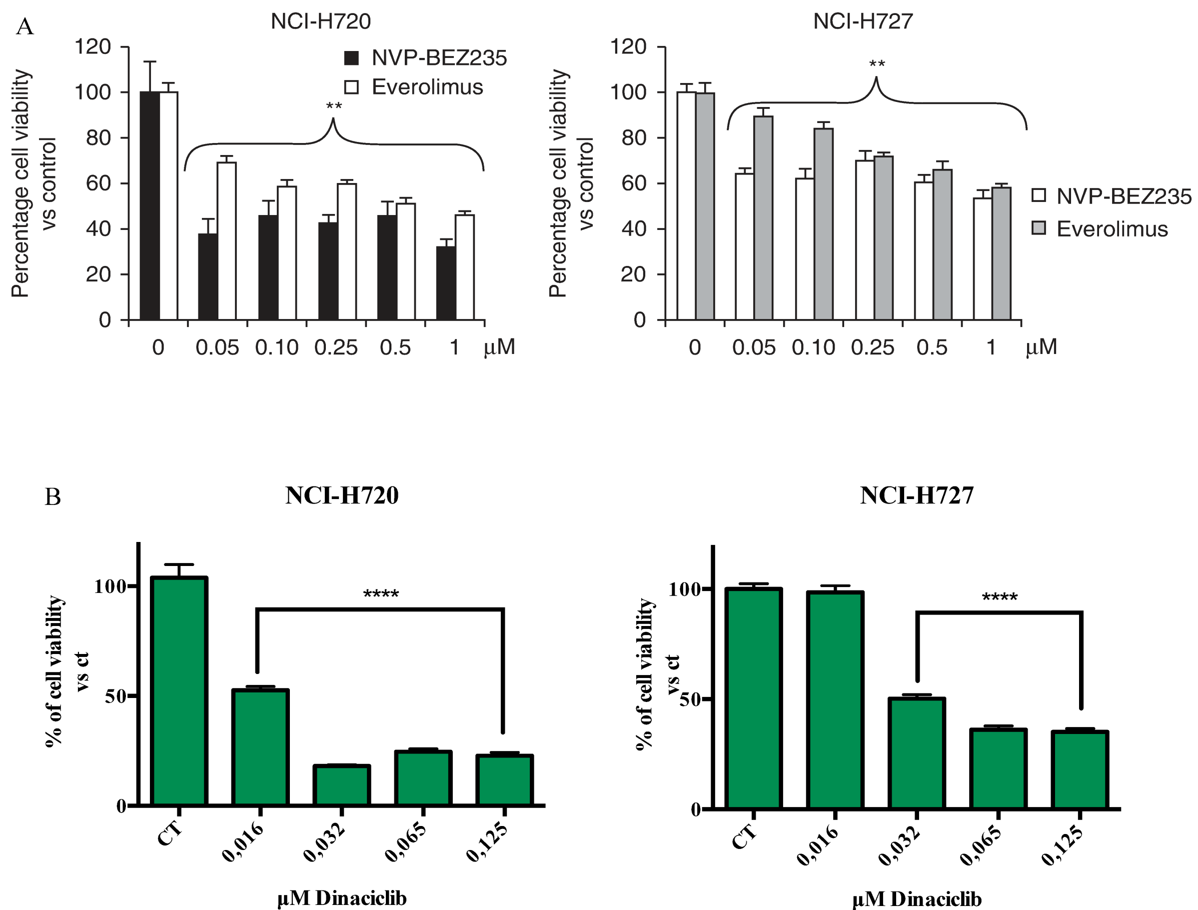

Figure 6: *previous results obtained in our lab, published (A) and unpublished (B) studies, concerning Everolimus and Dinaciclib action on NCI-H720 and NCI-H727 cells* [277].

2. AIM OF THE STUDY

NENs are a heterogeneous group of tumours originating from neuroendocrine cells distributed ubiquitously throughout the body [1, 196, 281]. Multiple therapeutic options are available for patients with NENs and surgical resection represents the first line treatment. However, since it is not feasible in most of the cases, medical therapies have gained a key role in the clinical management of NENs [282, 283, 284]. In particular, molecular target therapies have acquired in latest years a growing importance in NENs management and actually two agents are approved for NENs therapy: Everolimus, an mTOR inhibitor approved for the treatment of both pNENs and BP-NENs, and Sunitinib, a multiple RTKs inhibitor approved for pNENs treatment [188, 285]. Despite these agents have shown to improve PFS, mechanism of acquired resistance may occur leading to the need of new approaches. This is particularly true especially for NENs affecting the broncho-pulmonary tract that, at the moment, can count on few therapies for their management [197, 286, 287]. In recent years the study of malignancies through new technologies gained a lot of importance, exploring innovative systems and approaches in order to take in account all tumour features. Recent research insights should be exploited and, in order to find new molecular targets and understand drug resistance mechanisms, three dimensional tumour structure as well as tumour cells heterogeneity should be taken into consideration [264, 275]. Therefore, this study has several parts all linked together in a common aim: the improvement of our knowledge about NENs biology and drugs resistance in order to find effective approaches for their management. Detailed aims are indicated below.

- Evaluation of 3D culture methods for neuroendocrine neoplasms study: 3D cultures represent a useful method in the attempt to fill the gap between *in vitro* and *in vivo* systems and, therefore, obtain more reliable experimental results. In order to understand which available culture method could represent the best option in terms of experimental easiness and reproducibility for NENs culture we have analysed three different "scaffold free" 3D culture methods. Therefore, we have tested Sunitinib at different concentrations on 3D spheroids obtained from a pNEN cell line, the BON1 cells, generated with different

methods. We have then evaluated the results of each 3D method according to their different specific features and tried to identify which method can represent the best option to study drug activity in BON1 cells.

- Identification of new putative molecular targets in brocho-pulmonary neuroendocrine neoplasms through the use of 2D and 3D cultures: BP-NENs are neoplasms still lacking of a specific medical therapy and, despite Sunitinib role in the treatment of pNENs, there is no clear evidence of its efficacy in BP-NENs. Therefore, new *in vitro* studies can be useful to understand the possible role of Sunitinib in BP-NENs treatment and find new potential molecular targets. Therefore, our aim was to elucidate Sunitinib mechanism of action in two BP-NEN cell lines and primary cultures, in order to identify new potential therapeutic targets in the treatment of this malignancy.
- New approaches in broncho-pulmonary neuroendocrine neoplasms studies, from the comprehension of tumour microenvironment influence on tumour behaviour and response to drugs, to the opening of new frontiers in medical treatment: BP-NENs resistance to medical therapies can be due to several factors and, thanks to new upcoming evidence, we have explored the possible role of TME in BP-NENs behaviour and drug resistance through the use of a lung fibroblast cell line. Therefore, we have cultured in 2D and in 3D two BP-NEN cell lines, NCI-H720 and NCI-H727, both in co-culture with fibroblasts or in presence of their conditioned medium. The effects of fibroblasts conditioned medium and co-culture have been evaluated in terms of response to tested drugs and spheroids formation. Beyond Everolimus effects, we have also analysed the action of Dinaciclib, a cyclines and CDKs inhibitor, in order to understand whether its action could overcome the previously reported resistance to Everolimus displayed by NCI-H720.

3. MATERIALS AND METHODS

3.1 IMMORTALIZED HUMAN CELL LINES

BON1 cells, derived from human pNEN, were a kind gift from Dr. C. (Auernhammer, Medizinische Klinik II, University of Munich, Germany). NCI-H727 and NCI-H720 cell lines, derived from human BP-NEN, and MRC5, human lung fibroblast cell line, were purchased from the American Type Culture Collection (ATCC). BON1 cells were grown in 1:1 mixture of F12K and DMEM medium (Euroclone, MI, Italy), while NCI-H727 and NCI-H720 cell lines were cultured in RPMI-1640 medium (Euroclone, MI, Italy) and MRC5 were grown in Eagle's Minimum Essential Medium EMEM (ATCC Manassas, VI, US). All mediums were supplemented with 10% fetal bovine serum (FBS), 10 U/ml Penicillin/Streptomycin, referred as “complete medium”, and cultured at 37°C in a humidified atmosphere with 5% CO_2. Experiments were performed within the seventh passage.

3.2 HUMAN BRONCHOPULMONARY TISSUE COLLECTION AND PRIMARY CUTURES

BP-NEN samples were used in the second part of the study. Samples derived from nine patients, whose characteristics are shown in table 6 page 102 (four females and five males; age = 21/81, mean = 50.44; ES ± 7.14). All patients went though surgical resection and were diagnosed, after histological and immunohistochemical confirmation, for BP-NENs according to the current WHO classification. Primary cultures were obtained in the following way. Upon arrival in the lab, a portion of the fresh tissue was immediately minced in serum- free RMPI-1640 medium under sterile conditions. Tissues were washed several times with 0.9% NaCl solution, and after removing the majority of physiological solution, were dissected into small pieces with sterile scalpels. The tissue pieces were incubated with 2.5% trypsin in Hanks' Balanced Salt Solution (HBSS) (Euroclone

Ltd., Wettherby, UK), with 0.3% collagenase (Worthington Biochemical Corporation, Lakewood, NJ, USA), and 5 ml of serum-free RMPI-1640 medium in Orbital Shaking Incubator SI50 (Bibby Scientific Limited, Beacon Road, Stone, Staffordshire, UK) at 37 °C for 60 min. The volumes of these substances were chosen depending on the size and characteristics of each tissue sample. Cell suspensions were filtered through syringes and needles of decreasing sizes in order to obtain a better tissue disaggregation. Subsequently, the cell suspension was centrifuged at 800 g, for 5 min at 4°C. After supernatant discard, tumour cells were resuspended in complete medium, seeded in 96- well black plates ($1x10^4$ cells/well) and incubated at the same condition as immortalised cell lines. After approximately 24 hours, cells were treated with Sunitinib 5µM, Linsitinib 5µM, Erlotinib 5 µM, IGF1 100nM, EGF 30nM and VEGF 50ng/ml alone and in combination with further evaluation of cell viability and/or caspase 3/7activity. Experiments were performed within 3 days in order to prevent fibroblast overgrowth, which is commonly observed after 4 days of culture. All the experiments that involved primary cultures were in accordance with the regulation of the University of Ferrara and approved by the University of Ferrara Ethics Committee. Informed consents were obtained for disclosing clinical investigation and performing the *in vitro* study, in accordance with the local ethical committee.

3.3 DRUGS AND CHEMICALS

Sunitinib, Erlotinib, Linsitinib, Everolimus and Dinaciclib were purchased from Selleckchem (TX, USA). All the compounds were dissolved in dimethyl sulfoxide (DMSO) and Sunitinib, Erlotinib, Linsitinib and Dinaciclib were stored at −80°C as 10 mM stock solutions while Everolimus was stored at -20°C as 100 µM stock solution.

EGF and IGF1 were purchased from ProSpec protein specialists (East Brunswick, NJ, USA); VEGF was purchased from Peprotech Inc (Rocky Hill, NJ, USA). EGF, IGF1 and VEGF were

resuspended in sterile PBS with 0.1% BSA. All other reagents, if not specified, were purchased from Sigma Aldrich (St. Louis, MI, USA).

3.4 3D CELL CULTURE METHODS

3D spheroids were obtained using three different methods. The first two methods were employed in the first part of the study while the third method was used in all the three part of the study.

The first method employed 96-well hanging drop plates (Perfecta ® 3D, 3D Biomatrix, MI, USA). BON1 cells were seeded at 2.4×10^3 cells/well in 30 μl/well complete medium and allowed to form compact 3D aggregates. Two days after seeding and spheroids formation, aggregates were moved into another 96-well plate and treated through the addiction of 70 μl of fresh complete medium with Sunitinib at 2.5, 5, and 7μM into each well. Pictures were taken before treatments addiction and before MTT solution addiction for cell viability assessment.

In the second method, 500 BON1 cells were seeded in a 24-well plate with a repellent surface (CELLSTAR® Cell-Repellent Surface, greiner bio-one, KR, AU) and left on a microplate mixer overnight at 80 rpm. Medium was removed the 4th day after seeding and cells were treated with Sunitinib 1, 2.5, and 5 μM. 3 days after, treatments were renewed in new fresh complete medium. Pictures were taken before adding treatments and then at day 7 and 10 after treatment.

In the third method, 30 μl complete medium containing 2.4×10^3 cells were seeded in each well in an ultra low attachment 96-well plate (Corning® 96-wellClearRound Bottom Ultra-Low Attachment Microplate, NY, USA (ULA plates)). After seeding, the plates were centrifuged at 300*g* for 3min and treated 72h later. This method was used to seed BON1 cells in the first part and NCI-H720 / NCI-H727 in the second and third part. Moreover, in the third part, NCI-H720 / NCI-H727 were also seeded in co-culture with MRC5. In order to seed cells in co-culture, cancer cells and fibroblasts were seeded together keeping constant final concentration and volume as indicated before. Spheroids obtained with this culture method were treated with different compounds

according to the part of the study. Treatments were performed adding 70 µl of fresh complete medium supplemented with the indicated compounds and pictures were taken before treatments and CellTiter Glo solution addiction for cell viability assessment.

3.5 3D SPHEROID SIZE EVALUATION

Spheroid size was evaluated in the first part of the study by measuring BON1 spheroid perimeter with Image J software (NIH, Bethesda, Maryland, USA). Results are expressed as mean pixel measure ± S.E.M. vs. vehicle- treated control cells from three independent experiments in two replicates. Pictures for spheroid size evaluation were taken with a Zeiss Axiovert 200/M-based phase-contrast microscope for cells seeded with the 24-well plate with a repellent surface while, for spheroids seeded with the 96-well hanging drop plates and the ULA plates, pictures were taken using EVOS FL Cell imaging System (Thermo Fisher, Waltham, MA, USA).

3.6 IMMUNOHISTOCHEMISTRY

Immunohistochemistry (IHC) was performed in the first part of the study. BON1 spheroids were seeded in a 24-well plate with a repellent surface as indicated above and, after 9 days, fixed with 10% formalin, washed with 70% ethanol and embedded in HistoGel (Thermo Scientific, HG-4000-012). After that, spheroids were embedded in paraffin and cut in 3µm layer slides. Successively, sections were deparaffinized, dehydrated, and incubated with caspase 3 primary antibody (Cell Signaling, Danvers, USA) diluted 1:1,000 in 5% w/v nonfat dry milk, 1X TBS, 0.1% Tween® 20 and incubated overnight at 4°C. All slides were counterstained with eosin and coverslipped.

3.7 MTT ANALYSIS

Variations in BON1 cell viability were detected using MTT assay (Sigma) in the first part of the study. MTT assay detects NAD(P)H- dependent cellular oxidoreductase activity, which, according to international literature, mirrors cell viability. Cell viability detection is possible thanks to the reduction promoted from NAD(P)H- dependent cellular oxidoreductase. Indeed, this enzyme reduces the MTT 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltenyltetrazolium bromide (tetrazolium dye) into its insoluble formazan product. These products are subsequently solubilized thanks to a solubilisation solution (in our case DMSO) that is added to each well. The solubilisation solution dissolves the insoluble formazan products into a colored solution. The absorbance of the solution is then quantified with a spectrophotometer. BON1 cells were seeded as spheroids using the 96-well hanging drop plate / ULA plate and treated 3 days after seeding with Sunitinib at the indicated concentrations. Control cells were treated with vehicle solution alone (DMSO). Spheroids were incubated with Sunitinib for 3 days and then 10 µl of 5 mg/ml MTT solution were added to each well. After 1 day, 100 µl of MTT solvent were added to each well and plates were incubated for 4 h in order to solubilize formazan crystals. Absorbance at 570 nm was then measured with GloMax® Explorer Multimode Microplate Reader (Promega, WI, USA). Results are expressed as mean value ± S.E.M. percent optical density (OD) vs. vehicle-treated control cells from three independent experiments in six replicates.

3.8 LIVE/DEAD CELLS ASSAY

NCI-H720 and NCI-H727 cell death or viability was assessed in the second part of the study and determined using the Ready Probes Cell Viability Imaging Kit (Blue/Green) (Life Technologies, Carlsbad, CAL, US). BP-NEN spheroids were seeded using ULA plates and treated with Sunitinib

5µM, Linsitinib 5µM, Erlotinib 5 µM, IGF1 100nM, EGF 30nM and VEGF 50ng/ml 3 days after seeding. 72h after treatment, 100µL of cell stain mix (two drops of each stain per ml of culture media) was added to each well and incubated with spheroids for 15 min. The different luminescence was observed using the EVOS FL Cell imaging System and fluorescence intensity was quantified using Image J software.

3.9 KINASE ACTIVITY ASSAY

Primary cultures kinase activity assay was evaluated in the second part of the study. AlphaScreen SureFire assays (Perkin Elmer, MI, Italy) was used for the measurement of EGFR (p-Tyr1068) and IGFR (p-Y1135/1136), p-VEGFR 2 (Tyr1175) levels. Cells were seeded 2×10^4 each well in complete medium using a 96-well plate and, after overnight attachment, were incubated for 24h with or without Sunitinib 5µM and EGF 30 nM, alone and in combination, or Sunitinib 5µM and IGF1 100 nM, alone or in combination. AlphaScreen SureFire assay is a bead based technology that allows the detection of phosphorylated proteins in cellular lysates in a highly and quantitative assay. Sandwich antibody complexes, which are only formed in the presence of analyte, are captured by AlphaScreen donor and acceptor beads, bringing them into close proximity (figure 7). The excitation of the donor bead at 680 nm provokes the release of singlet oxygen molecules that triggers a cascade of energy transfer in the acceptor bead, resulting in the emission of light at 520-620 nm. Light emission was measured with Envison plate reader (Perkin Elmer) using standard AlphaScreen settings. Outputs were recorded as counts per second (cps).

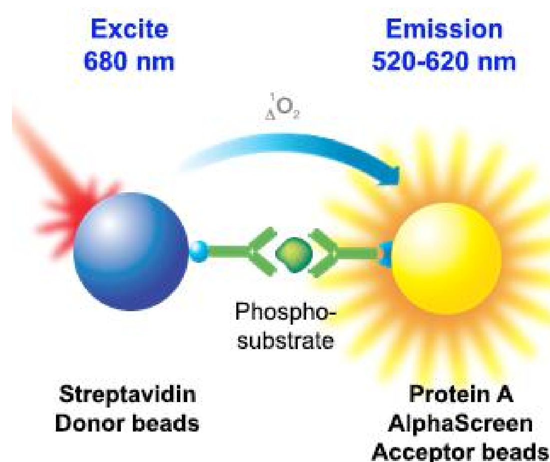

Figure 7: *AlphaScreen SureFire Assay Principle.*

3.10 CASPASE ACTIVATION

NCI-H720 and NCI-H727 caspase activity was assessed in the second part of the study and measured using Caspase-Glo 3/7 assay (Promega). The Caspase-Glo® 3/7 Assay is a homogeneous, luminescent assay that measures caspase-3 and -7 activities. This assay provides a proluminescent caspase-3/7 DEVD-aminoluciferin substrate and a proprietary thermostable luciferase. The reagent promotes cell lysis and, therefore, the subsequent caspase cleavage of the substrate. This process promotes the formation of free aminoluciferin that is consumed by luciferase generating "glow-type" luminescent signal, directly proportional to caspase-3/7 activity. Briefly, 2×10^4 cells/well were seeded in 96-well black plates and treated with Sunitinib, Linsitinib, Erlotinib, IGF1, VEGF and EGF at the previously indicated concentration. Cells were incubated with the compounds for 72 h. Subsequently, the Caspase-Glo 3/7 reagent was added directly to the cell culture plates, which were then shaken at 12.7*g* for 30s, incubated at room temperature for 1h and measured for luminescent output (relative luminescence unit (RLU)). Envision Multilabel Reader was used for the measurement and results are expressed as mean value ± S.E.M. percentage RLU vs. vehicle-treated control cells from three independent experiments in six replicates.

3.11 CELL VIABILITY ASSAY

NCI-H720 and NCI-H727 viable cell number variations were assessed with the CellTiter Glo Luminescent Cell Viability Assay (Promega) in the second and third part of the study. This assay is a homogeneous method to determine the number of viable cells in culture and is based on ATP quantitation present in the well. ATP is considered as a signal of metabolically active cells and can therefore represent a good indicator for viability assessment. The Assay relies on the properties of a proprietary thermostable luciferase that, in presence of its substrates, generates a stable “glow-type” luminescent signal. Light emission can be measured through the use of a luminometer and, in this setting, it is directly proportional to the amount of ATP inside the well and, therefore, to viable cells. The detailed process is indicated in figure 8. This assay was used to analyse variation in cell viability in both the second and the third part of the study.

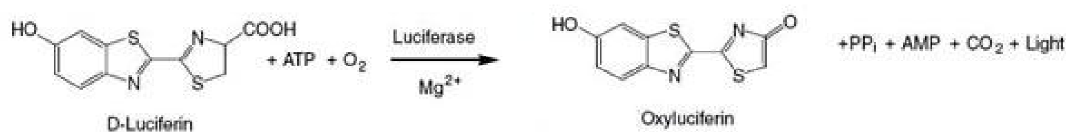

Figure 8: *The luciferase reaction. Mono-oxygenation of luciferin is catalysed by luciferase in the presence of Mg2+, ATP and molecular oxygen.*

In the second part of the study 2×10^4 cells were seeded for each well in 96-well black plates as a monolayer and treated for 72h.

In the third part of the study this assay was used on cells cultured as a monolayer or spheroids.

- Monolayer: 2×10^4 cells were seeded for each well in 96-well black plates and treated with Everolimus 100 nM, Dinaciclib 50 nM and conditioned medium for 96h.
- Spheroids: spheroids were obtained with the third culture method indicated above (ULA plates) and treated with Everolimus 100 nM, Dinaciclib 50 nM and conditioned medium for 96h.

Treatments were performed alone, in conditioned medium or with conditioned medium only.

In all of the cases, control cells were treated with vehicle alone (DMSO). After incubation, the revealing solution was added, and the luminescent output (relative luminescence units (RLUs)) was recorded using the Envision Multilable Reader (Perkin Elmer). Results are expressed as mean value ± S.E.M percentage RLU vs. the vehicle-treated control cells from three independent experiments in six replicates.

3.12 CONDITIONED MEDIUM COLLECTION

Conditioned medium (CM) collection was performed in the third part of the study. 5 ml of low serum EMEM (5% FBS) were incubated with MRC5 cells for 48 hours and then collected by centrifugation at 4°C for 30' at 1800xg. Subsequently, medium was passed through a 0.22μm filter, aliquoted and stored at -80°C. Treatments with CM were performed using a 1:1 solution with complete RPMI while control cells were treated with a 1:1 mixture between complete RPMI and low serum EMEM.

3.13 CO-INCUBATION WITH TRANSWELL

Transwell co-incubation was performed in the third part of the study. NCI-H720 and NCI-H727 cells were seeded at 10^5 concentration in a six well plate in complete RPMI. 24 hours later, 5 x 10^4 MRC5 cells were seeded inside of a transwell in 2 ml of serum free EMEM. Control cells were enriched with 2 ml of serum free EMEM. Co-cultures and control cells were left in incubation for 96h and then collected for two different purposes: a subsequent seed into 96 well plates for drug testing and for protein collection before Western blot analysis.

3.14 LIPOPHILIC TRACER STAINING

NCI-H720, NCI-H727 and MRC5 cells were stained using two lipophilic tracers in the third part of the study. SP-DiOC18(3) (Thermo Fisher), green fluorescence, was used for fibroblasts staining while Dil Stain (Thermo Fisher), red fluorescence, was used for cancer cells staining. These tracers diffuse laterally to stain the entire cell and are weakly fluorescent until incorporated into membranes. 5 µl of tracers were added to 10^6 cells suspension in serum free medium and then incubated at 37°C for 1h. After incubation, cells were centrifuged for 5 minutes at 300xg and washed twice with PBS. Subsequentely, cells were resuspended in fresh complete medium and seeded as spheroids as described above. Cells stained with lipophilic tracers were monitored for the first three days after seeding using JuLI™ Stage Real-Time Cell History Recorder (Nano-Entek, Waltham, MA, USA). Spheroids pictures were taken at day 3 after seeding with Zeiss Axiovert 200/M-based phase-contrast microscope (5 × objective).

3.15 PROTEIN ISOLATION AND WESTERN BLOT ANALYSIS

Protein isolation and Western blot (Wb) analyses were performed in the third part of the study. Protein extracts were obtained from NCI-H720 and NCI-H727 cells co-cultured, by transwell use, with MRC5 cells, as described above. Cancer cells and fibroblasts were incubated together for 96h and then cancer cells were collected in cold PBS through centrifugation. Dry pellets were dissolved in RIPA buffer (Pierce Biotechnology, Rockford, IL, USA) (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS), kept in ice for 1h, and then centrifuged at 13,000 rpm for 30 min at 4°C. The supernatant, containing the proteins, was then transferred to a new tube and protein concentration was measured by using the BCA Protein Assay Reagent Kit (Pierce Biotechnology).

For protein evaluation, proteins were mixed with 2X Laemmli buffer (62,5 mM Tris-HCl (pH 6.8), 25% glycerol, 2.1% sodium dodecyl sulphate, 0.01% bromophenol blue, DTT) and denatured at 95°C for 10' min. 30 μg of protein, deriving from human cell lines, were fractionated on a precast 4-12% SDS-PAGE gel (Perkin Elmer) and transferred by electrophoresis to polyvinylidene difluoride (PVDF) membranes (PerkinElmer) by using the Lightning BlotterTM (PerkinElmer). The membranes were incubated with the following antibodies: 1:1000 rabbit anti-human pmTOR (Cell Signaling Technology, Beverly, MA, USA); 1:1000 rabbit anti-human IGF1R (Santa Cruz Biotechnologies, Dallas, TX, USA); 1:1000 rabbit anti-human pIGF1R (Cell Signaling); 1:1000 rabbit anti-human EGFR (Cell Signaling); 1:1000 rabbit anti-human pEGFR (Cell Signaling); 1:1000 mouse anti-human CDK2 (ABCAM, Cambridge, MA, USA); 1:1000 mouse anti-human GAPDH (Cell Signaling). Anti-rabbit or anti-mouse HRP IgG antibodies (Dako Italia, Milano, Italy) were used at a dilution from 1:2000 to 1:5000 and proteins were revealed by enhanced chemiluminescence using the Azure c300 (Azure Biosystems, Dublin, CA, USA).

3.16 STATISTICAL ANALYSIS

Results are expressed as the mean ± S.E.M. Statistical analyses were carried out using ANOVA after proof of homogeneity of variances and normality tests, Tukey's test was used for multiple comparisons. Data were analysed using GraphPad (Prism v-7.0); *P* values <0.05 were considered significant (*). For all the other experiments, if not otherwise indicated, Student's paired or unpaired *t*-test was used to evaluate the individual differences between the means, and *P* values <0.05 were considered significant.

4. RESULTS

4.1 EVALUATION OF 3D CULTURE METHODS FOR NEUROENDOCRINE NEOPLASMS STUDY

4.1.1 INFLUENCE OF SUNITINIB ON BON1 SPHEROIDS CULTURED USING A 96-WELL HANGING DROP PLATE

BON1 cells were cultured using a 96-well hanging drop plate and treated, on the basis of previous experiments, with Sunitinib at different concentrations [288]. Spheroids pictures were taken before Sunitinib addition (3 days after seeding) and before MTT addiction (7 days after seeding). As shown in figure 9A, pictures taken at day 3, while spheroids were inside the hanging drop plate, are blurred and unclear. This is due to the plate peculiar conformation and to the fact that spheroids are in suspension inside a drop; all these features hamper the possibility to take clear and focused pictures. Pictures taken 7 days after seeding, later on spheroids transfer into a 96-well plate with ultra-low attachment bottom, appear clearer and more defined. This procedure implies several passages that amplify the likelihood of mistakes, prolongs hands-on time and increase experimental variability (Table 5, page 91). Spheroids perimeter analysis was performed both 3 and 7 days after seeding and is presented in figure 9B. This evaluation showed that there is no significant difference between mean perimeter of vehicle treated spheroids and mean perimeter of spheroids treated with different Sunitinib concentrations. These results are due to the great variability of the performed measurements, as indicated by a very high reproducibility error (see Table 5, page 91). Therefore, this method does not appear to be highly reproducible. Sunitinib effects on cell viability were assessed at day 7 through MTT analysis. As shown in figure 9C, treatment with Sunitinib significantly reduced cell viability by ~20% at all concentrations tested (P < 0.05 vs. vehicle-

treated cells).

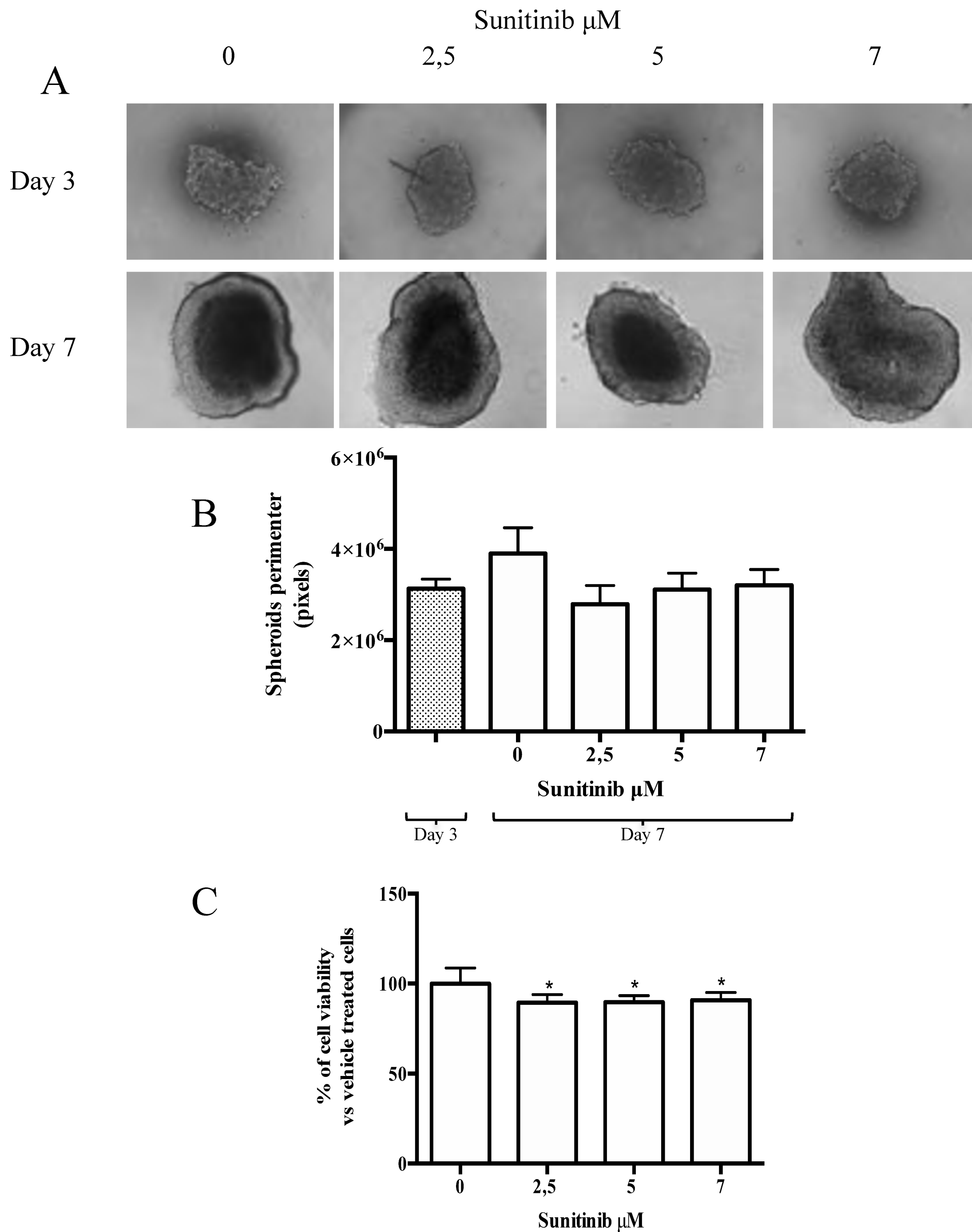

Figure 9: *Hanging drop method. BON1 cells were seeded into a 96 well hanging drop plate and treatments with Sunitinib were performed after spheroids transfer in a regular 96 well plate.* ***(A)*** *In the upper lane (Day 3) pictures were taken at day 3 after seeding inside the 96 hanging drop plate with EVOS FL Cell imaging System (10 × objective); in the lower lane (Day 7) spheroids pictures were taken at day 7 after transfer in a regular 96 well plate. Spheroids were treated at day 3, after transfer from the first to the second plate, with Sunitinib 2.5, 5, and 7 µM.* ***(B)*** *Perimeter analysis of spheroids was performed at day 3 and 7 and represented in a graph. Grey column: perimeter*

analysis at Day 3, before treatments. White columns: perimeter analysis at Day 7 under indicated treatments. The analysis was performed using Image J software and measurements were performed from three independent experiments in two replicates. ***(C)*** *Cell viability was measured as absorbance in three independent experiments with six replicates each, and it is expressed as the mean ± S.E.M. *P < 0.05 vs. vehicle cells* [289].

4.1.2 INFLUENCE OF SUNITINIB ON BON1 SPHEROIDS CULTURED USING A 24-WELL PLATE WITH A REPELLENT SURFACE

A 24-well plate with a repellent surface was employed for the generation of BON1 spheroids in order to compare the differences between 3D culture methods. Spheroids were seeded and then treated with different Sunitinib concentrations at day 4 and at day 7 after seeding. Pictures were taken before each treatment and before fixation for IHC studies. Moreover, changes in spheroids perimeter were evaluated in order to understand Sunitinib action on this type of 3D culture. Results indicate that at day 4 spheroids perimeter was highly homogeneous (figure 10A). Moreover, no detectable changes in spheroids size were observed at day 7 (figure 10B, left panel), while at day 10 after treatment with Sunitinib 1 and 2.5 μM spheroid perimeter decreased by ~ 13 and 15%, respectively ($P < 0.01$ vs. vehicle- treated cells) (figure 10B, right panel). Perimeter analysis could not be performed at day 10 for spheroids treated with Sunitinib 5 μM since they displayed an extremely irregular and loose shape. Furthermore, in order to explore additional possibilities offered by this culture method, Caspase 3 activation was evaluated by IHC analysis. As shown in Figure 10C, Caspase 3 IHC can be performed on spheroids treated with Sunitinib 1 and 2.5 μM. IHC of spheroids treated with Sunitinib 5 μM was not performed due to the complete spheroid disaggregation during the procedure. One of the most important risks related to the procedure is the inadvertently spheroids discard during the process. This high risk is due to the fact that this method needs medium refreshment (not only supplementation), which means that the medium has to be replaced at least twice before fixation. Reproducibility error could not be calculated due to the

loss of a high number of spheroids during the procedure (Table 5, page 91).

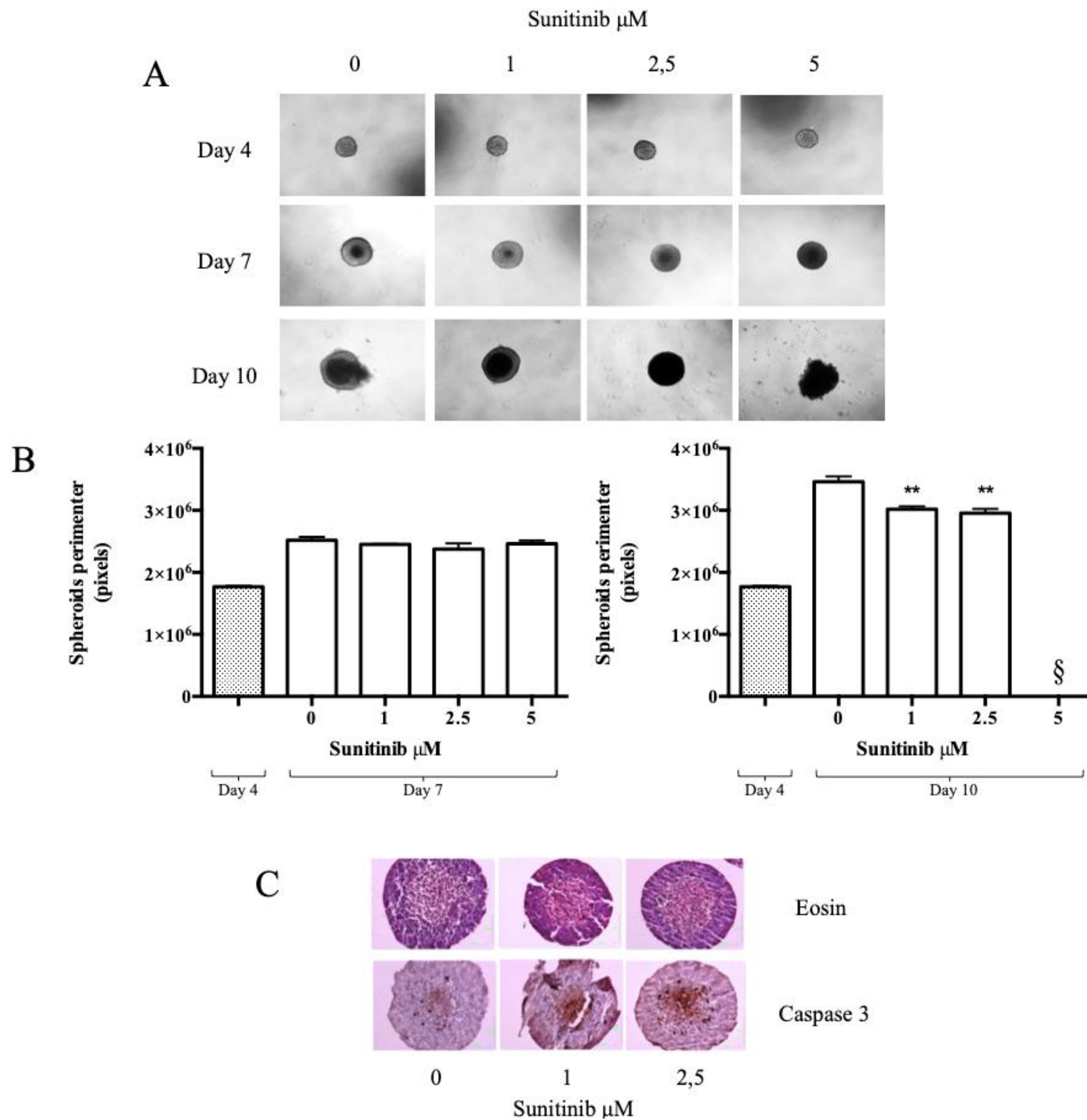

Figure 10: *24-well plate with a cell-repellent surface method. BON1 cells were seeded into a 24 well plate with a repellent surface, mixed overnight at 80 rpm.* ***(A)*** *Spheroids were treated with increasing Sunitinib concentrations and pictures were taken at day 4, 7, and 10 after seeding with a Zeiss Axiovert 200/M-based phase-contrast microscope (5 × objective).* ***(B)*** *Perimeter analysis of spheroids was performed at day 4, 7, and 10. Grey column: perimeter analysis at Day 4, before treatments. White columns: perimeter analysis at Day 7 and 10 under indicated treatments. The analysis was performed using Image J software and measurements were performed evaluating three independent experiments in two replicates. **$P < 0.01$ vs. vehicle cells at Day 10. § = 5 μM measurement was not detectable for technical reasons, as indicated in the results section.* ***(C)*** *Immunohistochemical expression of Caspase 3 in spheroids treated with different Sunitinib*

concentrations. Spheroids were fixed at day 10 and pictures were taken with a Zeiss Axiovert 200/M-based phase-contrast microscope. Pictures provide an overview of the entire spheroid stained with eosin and Caspase 3 antibody [289].

4.1.3 INFLUENCE OF SUNITINIB ON BON1 SPHEROIDS CULTURED USING AN ULTRA LOW ATTACHMENT 96-WELL PLATE

An ULA 96-well plate was also used to culture BON1 cells in order to further investigate the differences between available 3D culture methods. As shown in figure 11A, spheroids were treated with different Sunitinib concentrations and pictures were taken 3 and 7 days after seeding. In this case, thanks to plate conformation, pictures quality is improved and spheroids appear clear and focused. Spheroids perimeter was then investigated; we found a significant decrease in this parameter at day 7 after treatment with Sunitinib 2.5 μM by ~ 18% ($P < 0.05$ vs. vehicle-treated cells) and treatment with Sunitinib 5 and 7μM by ~ 21% ($P < 0.05$ vs. vehicle-treated cells) (figure 11B). Moreover, as shown in figure 11C, MTT assay showed that Sunitinib at 2.5, 5, and 7 μM significantly decreases cell viability by ~ 20% ($P < 0.01$ vs. vehicle-treated cells). In this method medium can be supplemented directly in culture wells without any need to spheroid transfer or medium refreshment. This means that there is a minor risk of spheroids discard. In addition, less replicates are sufficient and reduced incubation times are needed as compared to the previous methods in order to observe Sunitinib effects on BON1 spheroids. Performed measurements show that variability is reduced and this is underlined by a reproducibility error that is less than half as compared to that recorded for the first method (see Table 5 page 91).

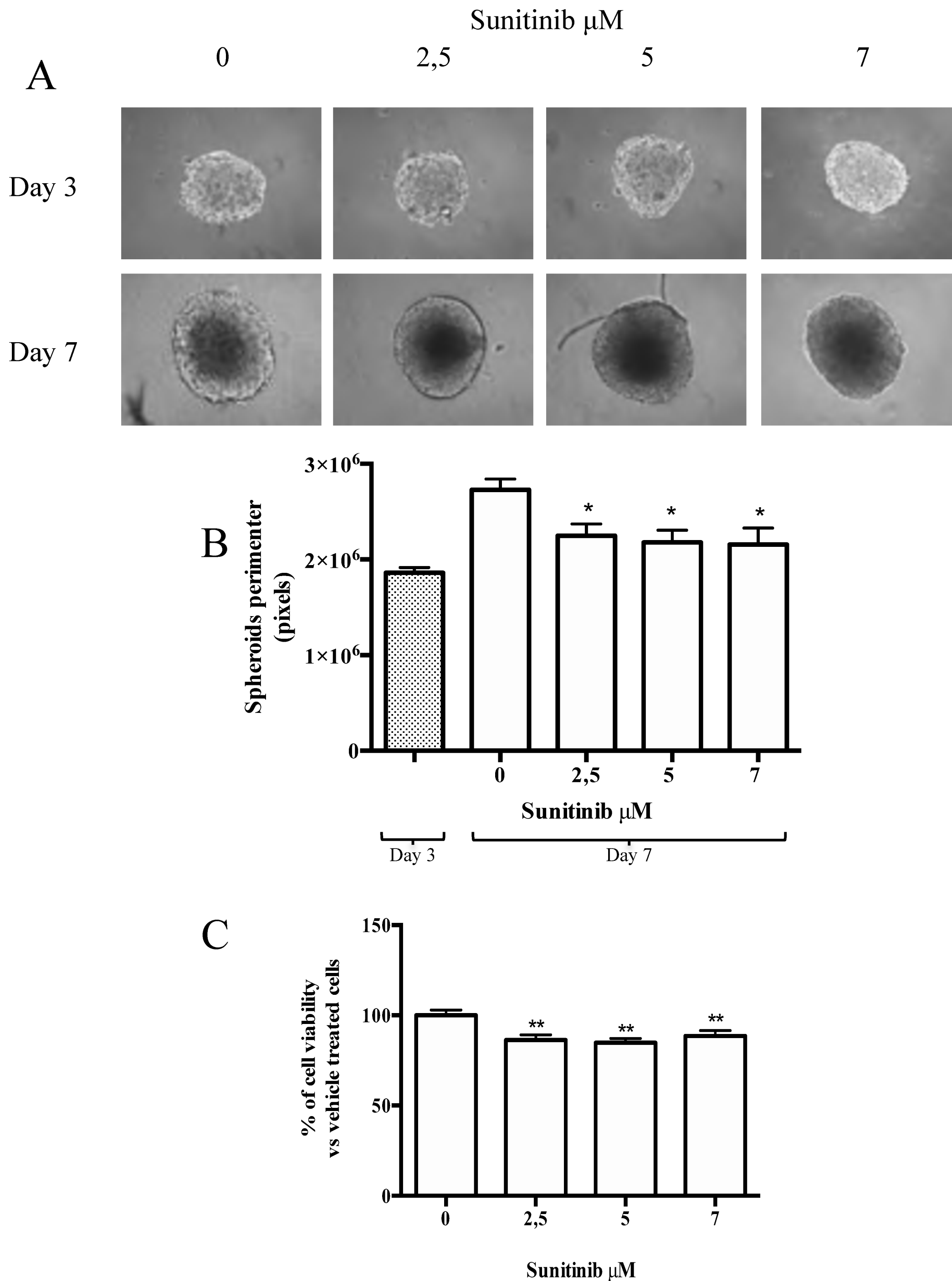

Figure 11: *ULA plate method. BON1 cells were seeded into an ultra low attachment 96 well plate and spheroids were obtained by centrifugation.* ***(A)*** *Spheroids were treated with increasing Sunitinib concentrations and pictures were taken with EVOS FL Cell imaging System (10 × objective) at Day 3 and 7 after seeding.* ***(B)*** *Perimeter analysis of spheroids was performed at Day 3 and 7. Grey column: perimeter analysis at Day 3, before treatments. White columns: perimeter analysis at Day 7 under indicated treatments. The analysis was performed using Image J software and measurements were performed from three independent experiments in two replicates. *P < 0.05 vs. vehicle cells.* ***(C)*** *Cell metabolic activity was measured as absorbance in three independent experiments with six replicates each, and it is expressed as the mean ± S.E.M. **P < 0.01 vs. vehicle cells [289].*

	3D Culture Methods		
	96-well hanging drop plate	**24-well plate with a cell-repellent surface**	**Ultra-low attachment 96-well plates**
Cell seeding	Easy	Easy	Easy
Easiness in spheroids formation	Easy	Intermediate	Easy
Morphology	Round type with jagged edges	Round-type	Round-type
Culture maintenance	Difficult	Difficult	Easy
Medium change	Difficult	Difficult	Difficult
Spheroids monitoring	Difficult	Intermediate	Easy
Picture quality	Low	High	High
Spheroid perimeter measurement reproducibility error	2,9 x 10^6 pixels	N. A.	1,2 x 10^6 pixels
Possibility of perform assays into the seeding plate	No	No	Yes
Overall time of the experiment	7 days	10 days	7 days

Table 5: *Comparison of the results obtained for 3D spheroids with the three different methods. Reproducibility error is not available for spheroids seeded with the second method since several spheroids were thrown away during medium refreshing* [(289)].

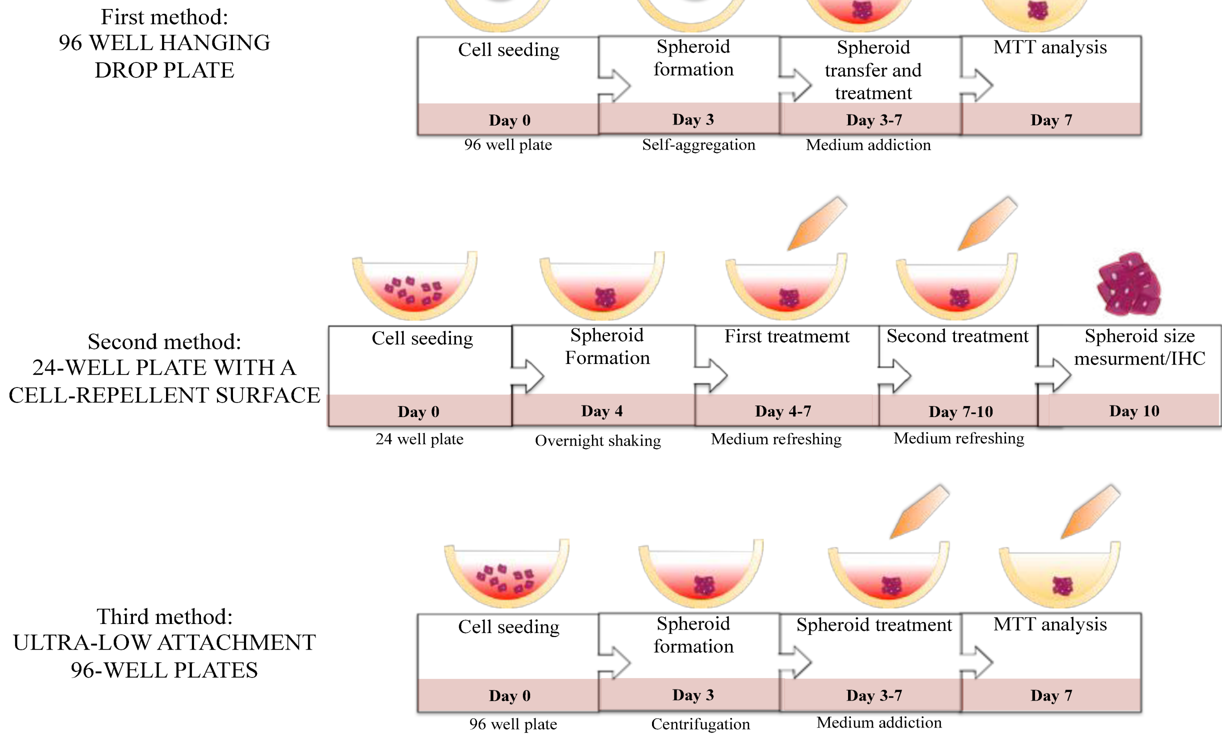

Figure 12: *Summary figure. Illustration of the steps of the different methods evaluated in the study* *(289)*.

4.2 IDENTIFICATION OF NEW PUTATIVE MOLECULAR TARGETS IN BRONCHO-PULMONARY NEUROENDOCRINE NEOPLASMS THROUGH THE USE OF 2D AND 3D CULTURES

4.2.1 INFLUENCE OF EGF, IGF1, VEGF AND SUNIINIB ON BP-NEN CELL LINES VIABILITY AND CASPASE ACTIVATION IN 2D AND 3D CULTURE SYSTEMS

We performed viability and caspase activation assays, through the use of 2D system culture model, in order to understand how treatments with Sunitinib and growth factors can influence cell viability of NCI-H720 and NCI-H727. Cancer cells were treated with EGF 30 nM, VEGF 50 ng/mL, IGF1 100 nM and/or Sunitinib 5 µM. Growth factors concentration was chosen on the basis patients reported plasma concentration while Sunitinib concentration was decided thanks to preliminary experiments (data non shown) [185]. As shown in figure 13A, EGF and VEGF did not affect cell viability of NCI-H720, while IGF1 significantly increased this parameter by 11% (P<0.05 vs. vehicle-treated cells). Sunitinib decreased cell viability by 40% ($P < 0.05$ vs. vehicle-treated cells). EGF and VEGF did not influence the effect of Sunitinib, while IGF1 was able to counteract the antiproliferative effects of Sunitinib (P<0.05 vs. Sunitinib- treated cells). In NCI-H727 cells, VEGF did not affect cell viability, while both EGF and IGF1 significantly increased this parameter by 10 and 15%, respectively (P<0.05 vs. vehicle-treated cells). Sunitinib decreased cell viability by 20% ($P < 0.05$ vs. vehicle-treated cells). VEGF did not influence the effect of Sunitinib that was instead decreased by co-treatment with IGF1 and EGF (P<0.05 vs. Sunitinib-treated cells).

Caspase 3/7 activation was further evaluated in order to verify if cell viability modulation could have been due to apoptosis process. As shown in figure 13B, in NCI-H720 cells, none of the growth factors was able to significantly affect caspase 3/7 activation while 5µM Sunitinib was able to increase the apoptotic activity by 100% (P<0.05 vs. vehicle-treated cells). On the other hand, this effect was partially affected by IGF1 (−34% vs. Sunitinib-treated cells). In NCI-H727 cells

(figure 13B), EGF was able to significantly reduce caspase 3/7 activity by 13%. Sunitinib increased the apoptotic activation by 22% ($P<0.05$ vs. vehicle-treated cells); however, this effect was significantly reduced by co-treatment with both EGF and IGF1.

BP-NEN cell lines were also cultured in 3D in order to observe if a more realistic solid tumour model could have influenced cells viability/ death. Changes were detected thanks to the use of two different fluorescent dyes. As shown in figure 13C, NCI-H720 and NCI-H727 spheroids treated with Sunitinib were characterised by a stronger green fluorescence, mostly focused in the middle of the spheroid, in comparison with cells treated with vehicle solution and growth factors. Green fluorescence represents cell death and its intensity evaluation overlaps with that obtained with cell viability assay (figure 13D). Green fluorescence predominance indicates a more extensive cell death in Sunitinib treated spheroids; this phenomenon is counteracted, at least in part, by co-treatment with growth factors especially in NCI-H727 spheroids.

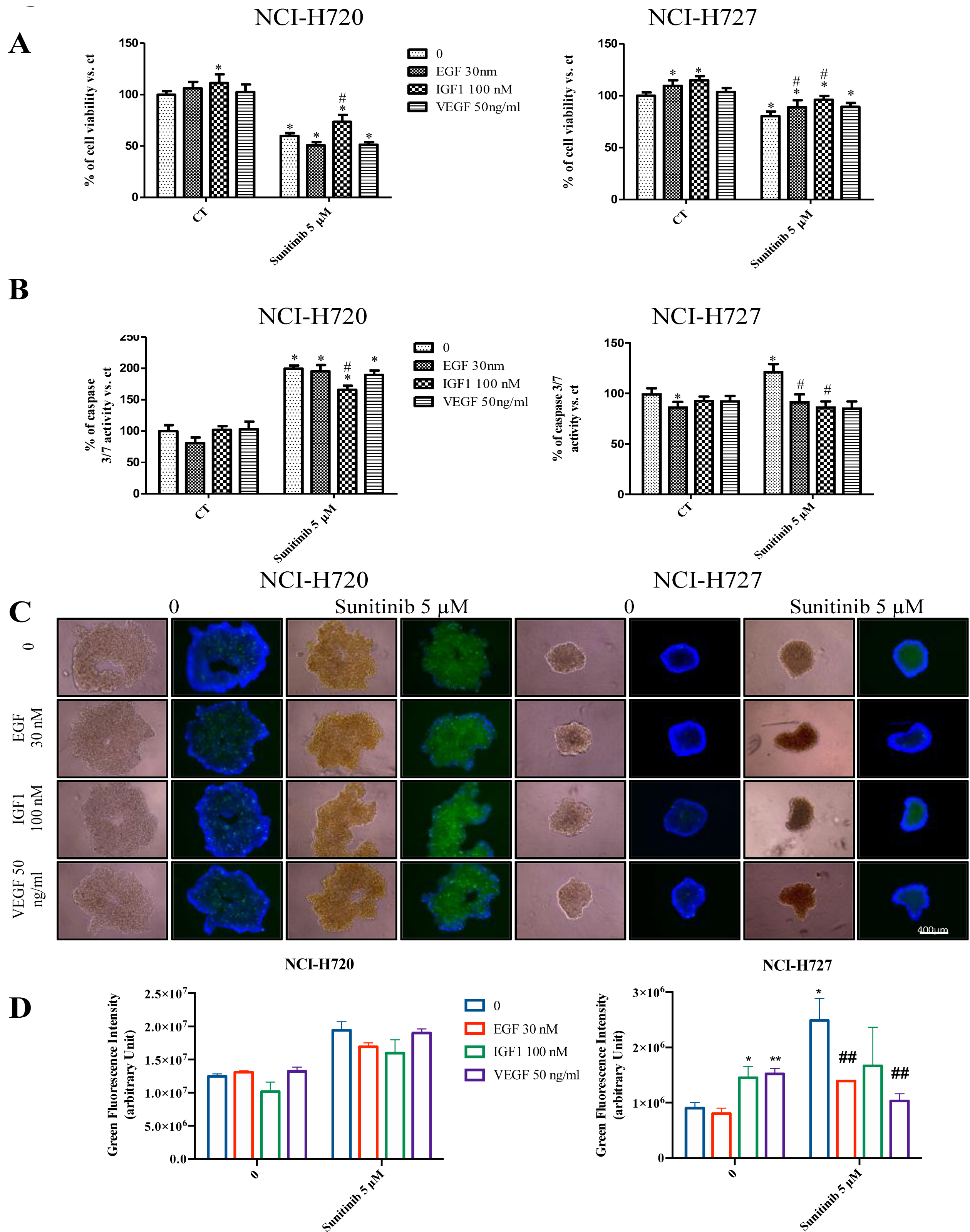

Figure 13: *Effects of Sunitinib and growth factors on cell viability, caspase activation and spheroids structure in human BP-NEN cell lines. NCI-H720 and NCI-H727 cells were incubated in 96-well plates for 72 h in culture medium supplemented with 5 µM Sunitinib or/plus IGF1 100 nM, EGF 30 nM and VEGF 50 ng/ml; control cells were treated with a vehicle solution. (A) Cell*

*viability was measured as luminescent output in three independent experiments with six replicates each, and it is expressed as the mean ± S.E.M. *P < 0.05 vs. vehicle cells; #P < 0.05 vs. cells treated with Sunitinib. (**B**) Caspase activity was measured as luminescent output in three independent experiments with six replicates each, and it is expressed as the mean ± S.E.M. *P < 0.05 vs. vehicle cells; #P < 0.05 vs. cells treated with Sunitinib. (**C**) Representative spheroids pictures were taken with EVOS FL Cell imaging System 72 h after treatment. NCI-H720 and NCI-H727 cells spheroids were treated as described earlier; pictures were taken without and with the fluorescent staining. The second and fourth columns in each plot represent the merge between the two fluorescence detected. The blue dye stains the nuclei of all cells (excitation/emission maxima: 360/460 nm), while the green dye stains only the nuclei of dead cells with compromised plasma membranes (excitation/emission maxima: 504/523 nm). (**D**) Green fluorescence from spheroids was analysed using Image J software and was measured as arbitrary units in three independent experiments and it is expressed as the mean ± S.E.M. *P < 0.05 vs. vehicle cells; **P < 0.01 vs. vehicle cells; #P < 0.05 vs. cells treated with Sunitinib; ##P < 0.01 vs. cells treated with Sunitinib.*

4.2.2 INFLUENCE OF EGFR, IGF1 AND SUNITINIB ON BP-NEN PRIMARY CELL LINES VIABILITY AND CASPASE ACTIVATION

In order to further verify data obtained in immortalised cell lines, we tested the effects of EGF and IGF1 on cell viability in BP-NEN primary cultures. As shown in figure 14A, even if results are not significant, we can observe that EGF 30 nM slightly increases cell viability, and partially counteracts the effects of Sunitinib in reducing this parameter. Due to the paucity of samples, statistical significance was not reached. However, this trend could be confirmed by further experiments including a higher samples number. On the other hand, IGF1 (figure 14B) was able to significantly increase cell viability of BP-NEN primary cells ($P < 0.05$) and to counteract, at least partially, Sunitinib effects. Concerning apoptotic activation (figure 14C and D), the caspase 3/7 activation promoted by Sunitinib 5μM, was significantly counteracted by both EGF and IGF1 ($P<0.00001$).

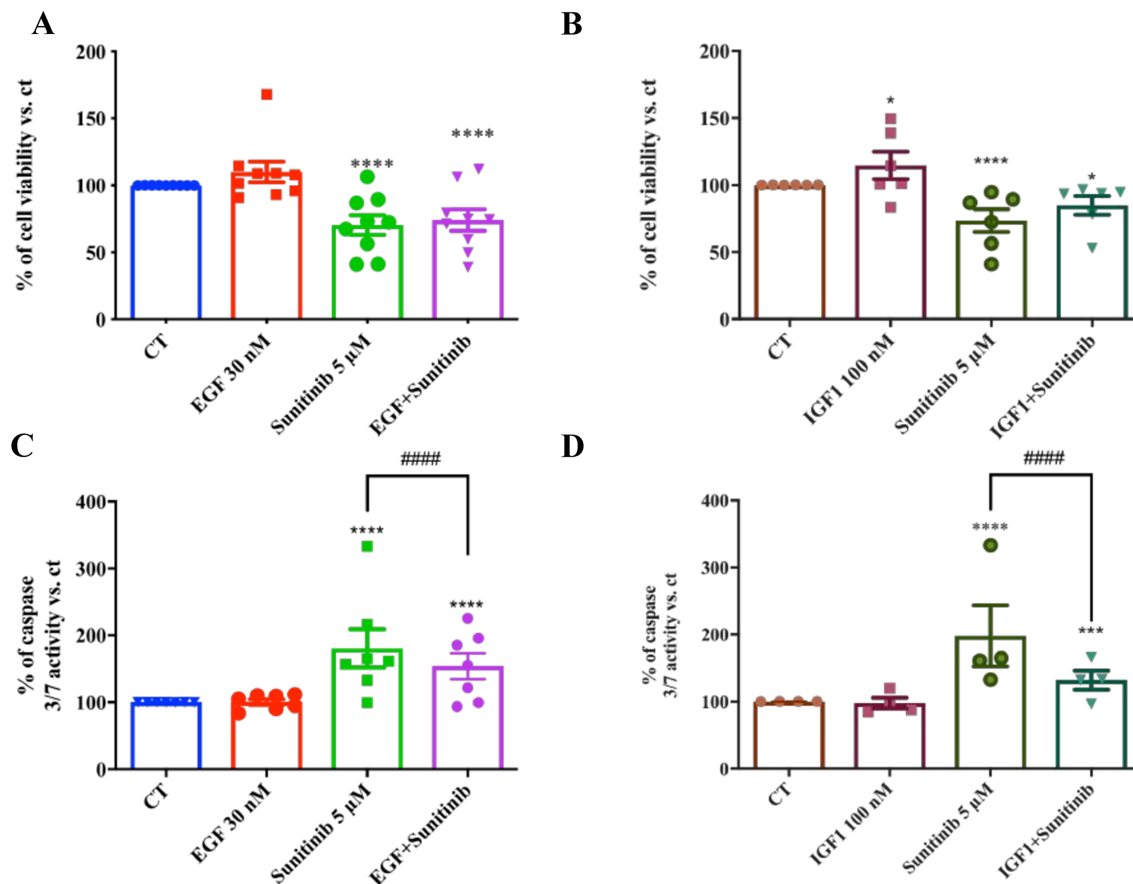

Figure 14: *Effects of Sunitinib and growth factors on cell viability and caspase activation in human BP-NEN primary cultures. Cells were derived from patients samples and incubated in 96-well plates for 48 h in culture medium supplemented with 5 µM Sunitinib or/plus EGF 30 nM (**A** and **C**) and IGF1 100 nM (**B** and **D**); control cells were treated with a vehicle solution. (**A** and **B**) Cell viability was measured as luminescent output and evaluated in one experiment with six replicates; each point represents the mean of single sample. Data are expressed as the mean ± S.E.M. *P < 0.05, ****P < 0.0001 vs. CT. (**C** and **D**) Caspase activity was measured as a luminescent output and evaluated in one experiment with six replicates each point represents the mean of single sample. Data are expressed as the mean ± S.E.M. ****P < 0.0001; ***P < 0.001 vs. CT; ####P < 0.00001 vs. cells treated with Sunitinib* **(Errore. Il segnalibro non è definito.)**.

4.2.3 EFFECTS OF SUNITINIB ON EGFR AND IGF1R PHOSPHORYLATION

Phosphorylation levels of EGFR and IGF1R were evaluated by a kinase activity assay in order to further investigate the mechanisms of action by which Sunitinib exerts its effects on BP-NEN cells. EGFR phosphorylation results after treatments were not univocal (figure 15A). On the other hand, as shown in figure 15B, Sunitinib was able to significantly decrease IGF1R phosphorylation

levels and its co-treatment with the corresponding growth factor IGF1 partially restored receptor phosphorylation. Concerning the evaluation of pVEGFR2 levels a modest and not significant reduction was observed in all sample analysed after Sunitinib treatments.

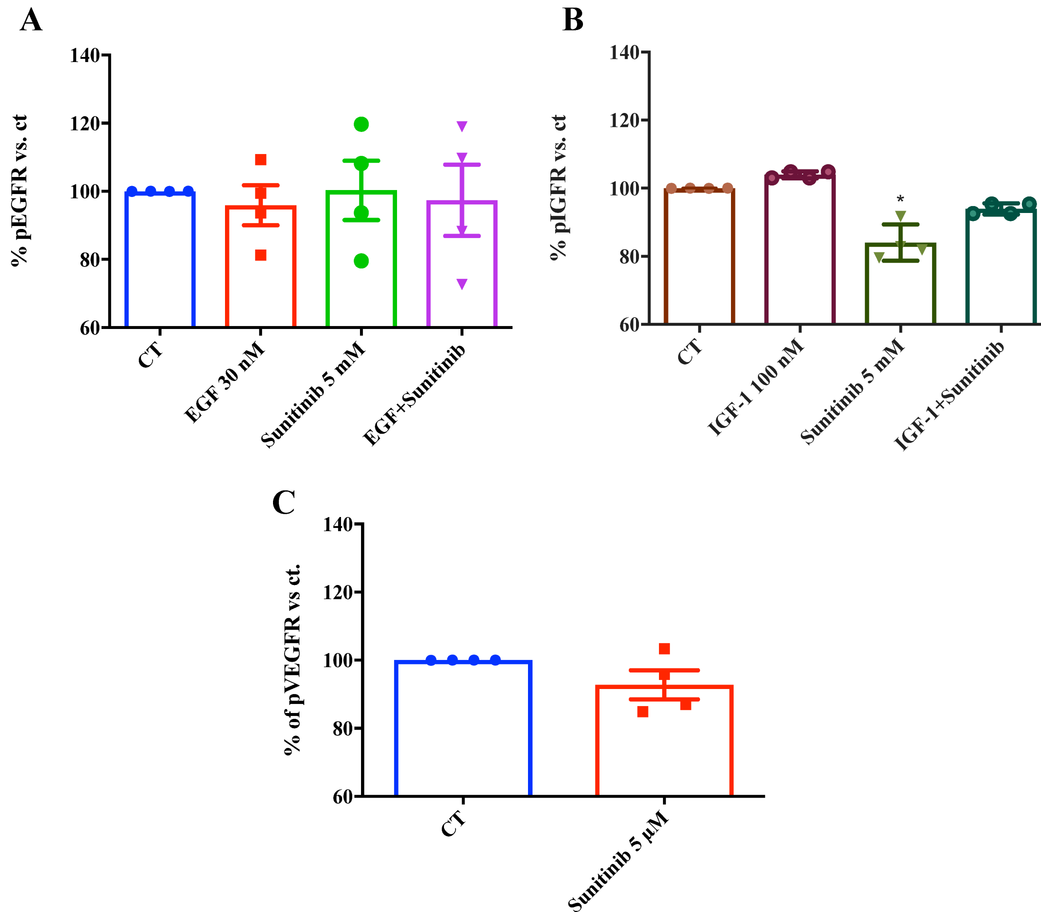

Figure 15: *Evaluation of phosphorylated EGFR and IGF1R in primary cultures after treatments. (**A** and **B**) Cells were treated with Sunitinib 5 µM or/plus EGF 30 nM (**A**) and IGF1 100 nM (**B**). Cells were incubated for 24 h and control cells were treated with a vehicle solution. (**A**) Primary culture cells were lysed and processed for phosphorylated EGFR SureFire assays in one experiment with three replicates; in each graph is shown the global mean. Data are expressed as percentage of p-EGFR vs. the control, considering as the control sample the untreated primary culture cells. (**B**) Primary culture cells were lysed and processed for phosphorylated IGF1R SureFire assays in one experiment with six replicates; in each graph is shown the global mean. Data are expressed as percentage of p-IGF1R vs. vehicle-treated cells. (**C**) Primary culture cells were lysed and processed for phosphorylated VEGFR2 SureFire assays in one experiment with six replicates; in each graph is shown the global mean. Data are expressed as percentage of p-VEGFR vs. vehicle-treated cells* **(Errore. Il segnalibro non è definito.)**.

4.2.4 INFLUENCE OF ERLOTINIB AND LINSITINIB ON BP-NEN CELL VIABILITY AND CASPASE ACTIVATION

Since our data indicate that EGF and IGF1 influence Sunitinib activity, we assessed cell viability and caspase activation using Erlotinib and Linsitinib, respectively an EGFR and IGF1R inhibitor, on NCI-H720 and NCI-H727 cells. Cells were treated with the two agents alone and in combination and, as shown in figure 16A, Erlotinib and Linsitinib displayed an antiproliferative action in both cell lines. Erlotinib decreased cell viability of both cell lines by ~ about a 20% ($P<0.01$ vs. vehicle-treated cells) while treatment with Linsitinib was able to reduce cell viability by 30% ($P<0.001$ vs. vehicle-treated cells). The combination of the two agents showed a greater effect in comparison with single treatments in both NCI-H720 and NCI-H727 cells reducing cell viability by ~ 40% vs. vehicle-treated cells and by 20% vs. treatment with Erlotinib ($P<0.05$ for NCI-H727 and $P<0.01$ for NCI-H720 cells). With regard to caspase activation, treatment with Erlotinib did not significantly affect apoptosis in NCI-H720 cells, while Linsitinib caused a significant increase by 40% ($P<0.01$% vs. vehicle-treated cells). The combination of the two agents strongly activated caspase causing an increase of the 250% vs. vehicle-treated cells ($P<0.01$).

In NCI-H727 cells, caspase activation was significant vs. vehicle-treated cells for both Erlotinib and Linsitinib alone. An increased caspase activation of 300% (P<0.01% vs. vehicle-treated cells) was observed when cells were treated with the combination of the two agents. In both cell lines, the combination of Erlotinib and Linsitinib, significantly improve the activation of caspase 3/7 as compare to both single agent ($P<0.001$).

Moreover, in order to observe the possible changes in cell viability/ death in a more realistic solid tumour model, we cultured BP-NEN cell lines in 3D using two different fluorescent dyes. As shown in figure 16C, NCI-H720 and NCI-H727 spheroids treated with Erlotinib and Linsitinib were characterised by a stronger green fluorescence in comparison with cells treated with vehicle solution. Cell death is indicate by green fluorescence and the evaluation of its intensity was

evaluated (figure 16D). Results analysis overlap with those obtained with cell viability assay. Furthermore, in order to confirm this data, we performed Erlotinib and Linsitinib treatment in BP-NEN primary cultures. Results indicate that both agents were able to reduce cell viability ($P < 0.01$) and to induce caspase 3/7 activation (significantly just for Erlotinib $P < 0.01$) (figure 17).

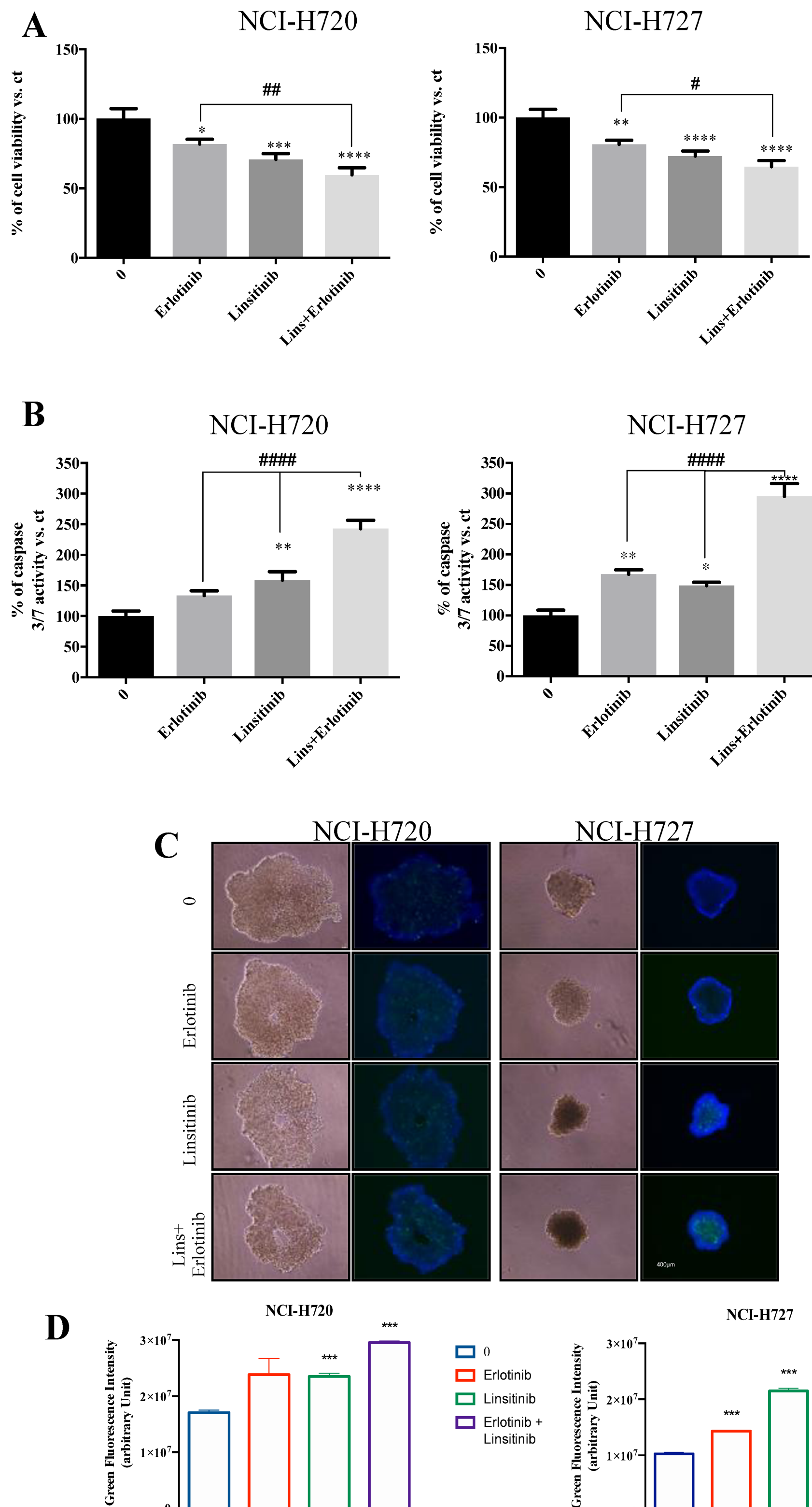

Figure 16: *Effects of Erlotinib and Linsitinib on cell viability, caspase activation and spheroids structure in human BP-NEN cell lines. NCI-H720 and NCI-H727 cells were incubated in 96-well plates for 72 h in culture medium supplemented with 5 µM Erlotinib and/or 5 µM Linsitinib; control cells were treated with a vehicle solution. (**A**) Cell viability was measured as luminescent output in three independent experiments with six replicates each, and it is expressed as the mean ± S.E.M. *P < 0.05 vs. vehicle cells or Erlotinib; **P < 0.01 vs. vehicle cells or Erlotinib; ***P < 0.001 vs. vehicle cells or Erlotinib; ****P < 0.0001 vs. vehicle cells or Erlotinib. (**B**) Caspases activity was measured as luminescent output in three independent experiments with six replicates each and it is expressed as the mean ± S.E.M. *P < 0.05 vs. vehicle cells or Erlotinib; **P < 0.01 vs. vehicle cells or Erlotinib; ***P < 0.001 vs. vehicle cells or Erlotinib; ****P < 00001 vs. vehicle cells or Erlotinib. (**C**) Representative spheroids pictures were taken with EVOS FL Cell imaging System 72 h after treatment. NCI-H720 and NCI-H727 were treated as described above; pictures were taken without and with the fluorescent staining. The second and fourth columns in each plot represent the merge between the two fluorescence dyes detected. The blue dye stains the nuclei of all cells (excitation/emission maxima: 360/460 nm) while the green dye stains only the nuclei of dead cells with compromised plasma membranes (excitation/emission maxima: 504/523 nm). (**D**) Green fluorescence from spheroids was analysed using Image J software and was measured as arbitrary units in three independent experiments and it is expressed as the mean ± S.E.M. **P < 0.01 vs. vehicle cells; ***P < 0.001 vs. vehicle cells.*

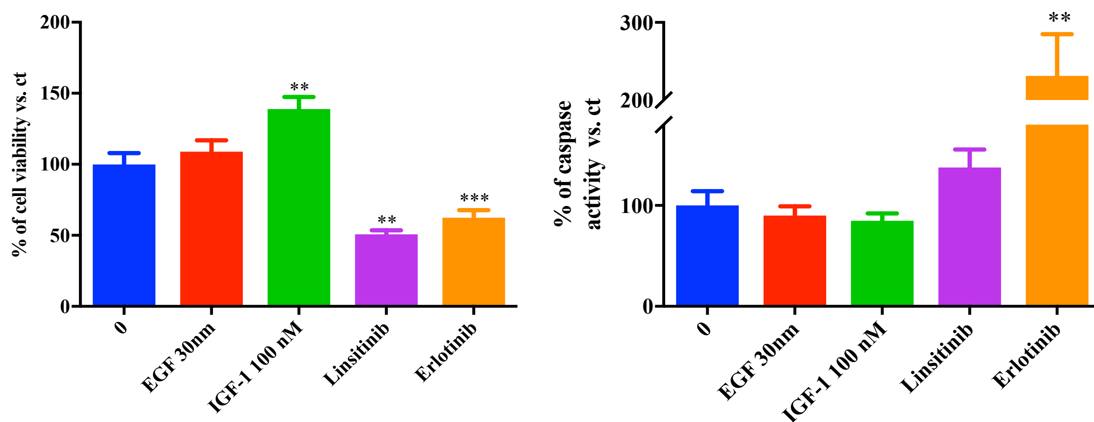

Figure 17: *Effects of Erlotinib and Linsitinib on cell viability, caspase activation in human BP-NEN primary culture. Cells were incubated in 96-well plates for 72h in culture medium supplemented with 5 µM Erlotinib, 5 µM Linsitinib, IGF1 100 nM or EGF 30 nM; control cells were treated with vehicle solution. (**A**) Cell viability was measured as luminescent output in three independent experiments with six replicates each, and it is expressed as the mean ± S.E.M. **P < 0.01, ***P < 0.001 vs. vehicle-treated cells. (**B**) Caspases activity was measured as luminescent output in three independent experiments with six replicates each and it is expressed as the mean ± S.E.M. **P < 0.01 vs. vehicle-treated cells* **(Errore. Il segnalibro non è definito.)**.

No.	Sex	Age	**Dimension** (mm)	Histology
1	F	61	190	Typical carcinoid
2	F	21	140	Typical carcinoid
3	M	24	300	Typical carcinoid
4	M	69	18	Typical carcinoid
5	M	43	350	Atypical carcinoid
6	M	33	120	Typical carcinoid
7	F	53	9 × 10 × 5	Typical carcinoid
8	F	69	22 × 7 × 20	Typical carcinoid
9	M	81	10 × 5 × 4	Atypical carcinoid
10	F	57	330	Typical carcinoid

Table 6: *BP-NENs patients* ***(Errore. Il segnalibro non è definito.)***.

4.3 NEW APPROACHES IN BRONCHO-PULMONARY NEUROENDOCRINE NEOPLASMS STUDIES, FROM THE COMPREHANSION OF TUMOUR MICROENVIRONMENT INLUENCE ON TUMOUR BEHAVIOUR AND RESPONSE TO DRUGS, TO THE OPENING OF NEW FRONTIERES IN MEDICAL TREATMENT

4.3.1 CO-CULTURE SPHEROIDS FORMATION

In order to study the possible relationship between TME and BP-NENs we started from the generation of a complex system. Therefore, cancer cells have been cultured in 3D and in co-culture with one of the main components of the tumour niche: fibroblasts. As shown in figure 18, cancer cells spheroids appear different depending on the cell line. NCI-H727 spheroids are compact and look as a single entity rather than cells near to one other. On the other hand, NCI-H720 cells do not form a proper spheroid and all the cells are all still visible. The situation appears completely different once the same are in co-culture with fibroblasts. Cancer cells cannot be identified in the spheroids that look more compact and solid. Moreover, in order to observe and understand spheroids formation, cancer cells and fibroblasts were stained with different lipophilic tracers. Cancer cells were stained with a red dye while MRC5 cell line was stained with a green dye. In figure 18 we can observe two different fluorescent stains and, thanks to the merge picture, we are also able to see the different cell lines arrangement in the spheroid. Fibroblasts seem to be mostly located in spheroids perimeter while cancer cells in the 3D aggregate centre. Furthermore, co-cultured spheroids formation has been recorded with a microscope in order to fully appreciate the process that leads to this complex system generation. Some of the crucial moments are represented in figure 19 for both NCI-H720 + MRC5 (figure 19A) cells and NCI-H727 + MRC5 cells (figure 19B).

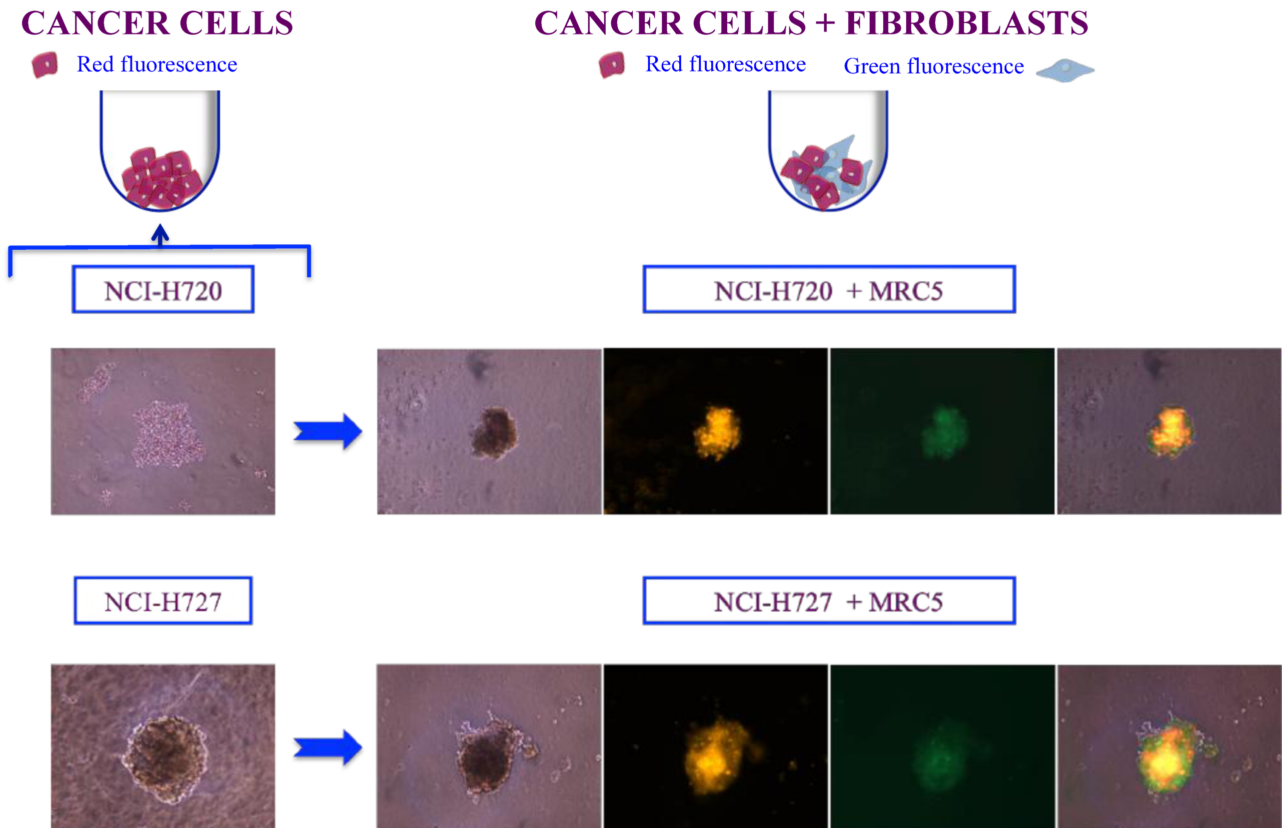

Figure 18: *Co-culture spheroids formation. NCI-H720 and NCI-H727 cancer cells were cultured as spheroids alone (left) or in co-culture with MRC5 lung fibroblast cells (right). In order to observe cells arrangement in the spheroid different cell types were stained using different lipophilic tracers. Cancer cells were stained with a red dye while fibroblasts with a green one. Pictures illustrate the different fluorescence alone and the merge image. Representative spheroids pictures were taken with EVOS FL Cell imaging System 72h after seeding.*

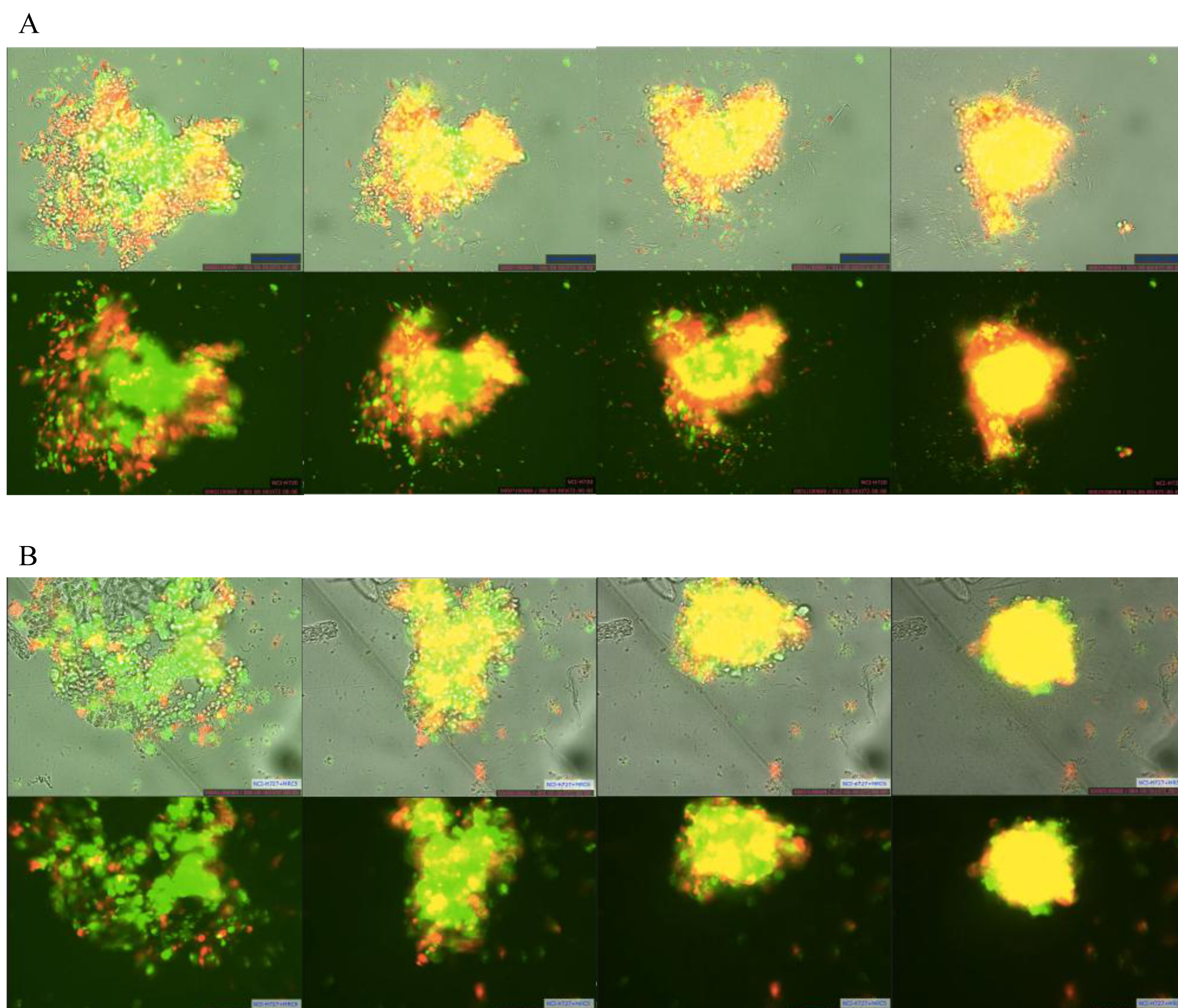

Figure 19: *Representative frames of the spheroids formation recorded video. Upper panel (**A**): NCI-H720 / MRC5 spheroid formation. Lower panel (**B**): NCI-H727 / MRC5 spheroid generation. The video was recorded with JuLI™ Stage Real-Time Cell History Recorder during the first 72h after seeding.*

4.3.2 INFLUENCE OF EVEROLIMUS AND DINACICLIB ON CELL VIABILITY OF BP-NENs CELLS CO-CULTURED WITH OR WITHOUT FIBROBLASTS

Since previous analysis in our lab demonstrated the effect of Everolimus and Dinaciclib on BP-NENs cells, we have investigated the action of these drugs also on cancer cells cultured as spheroids. Therefore, NCI-H720 and NCI-H727 spheroids were treated with Everolimus 100

nM and Dinaciclib 50 nM. Data indicate that both Everolimus and Dinaciclib have a strong effect in terms of cell viability reduction on BP-NENs cell lines (figure 20). Indeed, Everolimus decreases cell viability by 50% in NCI-H720 cells ($P < 0,0001$ vs. CT) while a 59% viability reduction is promoted by Dinaciclib ($P < 0,0001$ vs. CT). Moreover, Everolimus significantly decreases cell viability of NCI-H727 cells by 25% ($P < 0,01$ vs. CT) while Dinaciclib affects this parameter by 54% ($P < 0,0001$ vs. CT). Differences between Everolimus and Dinaciclib treatments are significant, indeed Dinaciclib promotes an further reduction cell viability by 11% ($P < 0,05$ vs. Everolimus) in NCI-H720 cells and by 29% ($P < 0,01$ vs. Everolimus) in NCI-H727 cells.

Furthermore, these treatments were repeated also for cancer cells co-cultured with the MRC5 cell line in order to understand whether fibroblast co-culture could modify the previously observed cell viability reduction. As shown in figure 20, treatments with Everolimus and Dinaciclib decrease cell viability also of cancer cells and fibroblasts spheroids. In particular, Everolimus decreases NCI-H720/MRC5 cell viability by 33% ($P < 0,0001$ vs. CT) and by 19% ($P < 0,05$ vs. co-cultured CT) in NCI-H727/MRC5 spheroids. On the other hand, Dinaciclib significantly decreases cell viability of NCI-H720/MRC5 spheroids by 50% ($P < 0,0001$ vs. CT) and by 58% ($P < 0,0001$ vs. CT) in NCI-H727/MRC5 spheroids. Also in this case, Dinaciclib promotes an additional 17% decrease in cell viability in comparison with Everolimus ($P < 0,0001$ vs. Everolimus) in co-cultured NCI-H720 cells and an additional 39% decrease ($P < 0,0001$ vs. Everolimus) in co-cultured NCI-H727 cells. Moreover, we compared cell viability results obtained on cancer cells spheroids without or with fibroblasts. Results indicate that both Everolimus and Dinaciclib reduce NCI-H720 cell viability when co-cultured with fibroblasts to a lesser extent as compared to “simple” cancer cells spheroids. In particular, after Everolimus and Dinaciclib treatment co-cultured cells reduced cell viability to a lesser extent, by 17% ($P < 0,01$ vs. cancer cells spheroids) and 10% ($P < 0,05$ vs. cancer cells spheroids) as compared to “simple” cell spheroids, respectively. On the other hand, tests on NCI-H727 cells did not show significant differences and observed cell viability reduction was the same

in the two cell culture systems.

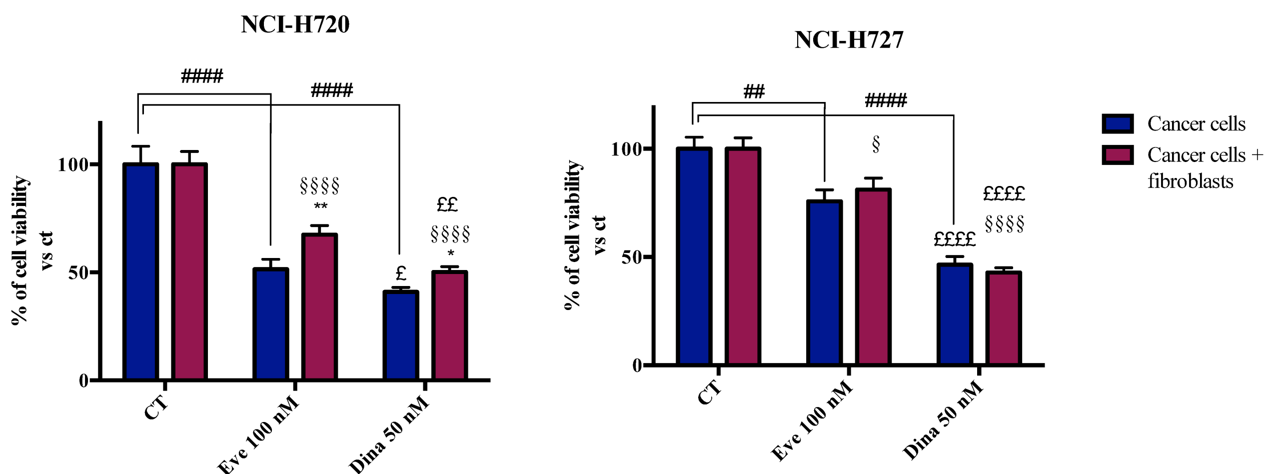

Figure 20: *Influence of Everolimus and Dinaciclib on cell viability of BP-NENs cells co-cultured and not with fibroblasts. NCI-H720 and NCI-H727 cells were cultured as spheroids alone (blue columns) and in co-culture with fibroblasts (red columns). Cells were seeded in ULA plates and, 72h after seeding, were incubated for 96h in culture medium supplemented with Everolimus 100 nM and Dinaciclib 50 nM; control cells were treated with a vehicle solution. Cell viability was measured as luminescent output in three independent experiments with six replicates each, and it is expressed as the mean ± S. E. M. £ P < 0,05 vs. cancer cells treated with Everolimus; ££ P < 0,01 vs. cancer cells treated with Everolimus; ££££ P < 0,0001 vs. cancer cells treated with Everolimus; #### P < 0,0001 vs. control cancer cells; § P < 0,05 vs. control cancer cells + fibroblasts; §§§§ P < 0,0001 vs. control cancer cells + fibroblasts; * P < 0,05 vs. cancer cells treated with Dinaciclib; ** P < 0,01 vs. cancer cells treated with Everolimus. Unpaired Student's T test was used for statistical analysis.*

4.3.3 FIBROBLASTS CONDITIONED MEDIUM INFLUENCE ON VIABILITY OF BP-NENs CELLS CULTURED IN 2D AND 3D

Since our aim was to investigate the possible influence of TME on BP-NENs we collected fibroblasts conditioned medium and we used it to treat cancer cells for 96h. Our aim was to detect the possible changes in cancer cells viability due to factors present in fibroblast conditioned medium. These tests were repeated both in 2D and in 3D and, as shown in figure 21A, conditioned medium was able to increase cell viability of 2D cultured NCI-H727 cells by 17% (P < 0,0001 vs.

CT). On the other hand, this effect is visible but not significant in NCI-H720 cells. However, the situation is different once conditioned medium was tested on cancer cells grown as spheroids. In this case, as shown in figure 21B, treatments with conditioned medium increase NCI-H720 cell viability by 15% (P < 0,05 vs. CT) while, on NCI-H727 cells, fibroblasts conditioned medium induce a greater increase in cell viability (77%; P < 0,0001 vs. CT).

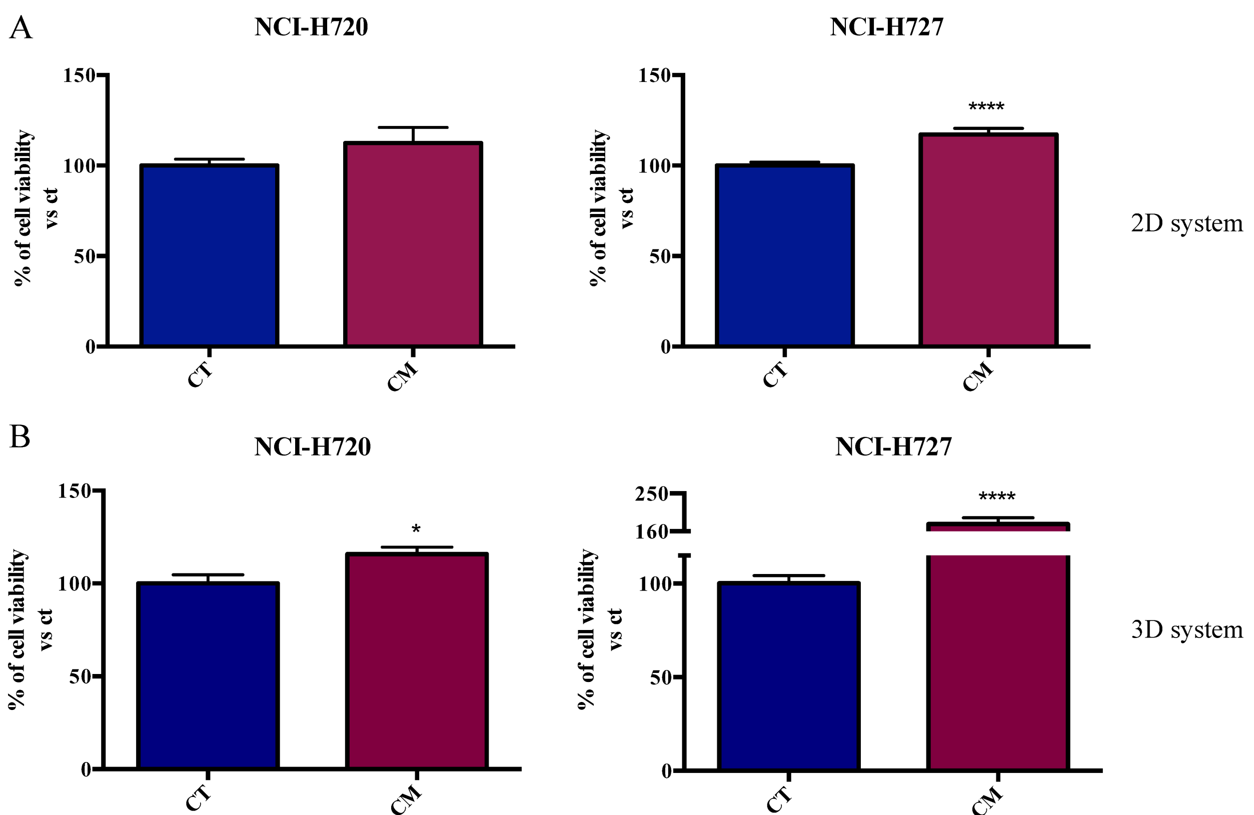

Figure 21: *Influence of fibroblasts conditioned medium on cell viability of BP-NENs cells cultured in 2D (**A**) and in 3D (**B**). Cells were seeded in black 96 well plates for 2D culture while for 3D culture cells were seeded in ULA plates. 2D cultures were treated the day after seeding while spheroids 72h after seeding. CT cells were incubated with low serum EMEM while cells were incubated with fibroblasts conditioned medium (CM) for 96h. Cell viability was measured as luminescent output in three independent experiments with twelve replicates each, and it is expressed as the mean ± S. E. M. * P < 0,05 vs. CT; **** P < 0,0001 vs. CT. Unpaired Student's T test was used for statistical analysis.*

4.3.4 INFLUENCE OF EVEROLIMUS AND DINACICLIB IN COMBINATION WITH FIBROBLASTS CONDITIONED MEDIUM ON 2D AND 3D BP-NENs CELLS VIABILITY

In order to further verify the effects of fibroblasts conditioned medium on cancer cells and assess its possible involvement in drug resistance, we tested Everolimus and Dinaciclib directly diluted in conditioned medium on cancer cells grown in 2D and 3D. Control cells were treated with a mixture of 1:1 complete RPMI and low serum EMEM. Results indicate that Everolimus diluted in low serum medium reduce cell viability of 2D NCI-H720 cells by 40% ($P < 0,0001$ vs. CT) while this parameter is reduced by 22% in 2D NCI-H727 cells ($P < 0,0001$ vs. CT) (figure 22A). In the same culture settings, Dinaciclib diluted in low serum generates a decrease in cell viability by 79% and 63% in NCI-H720 and NCI-H727 cells, respectively ($P < 0,0001$ vs. CT) (figure 22A). On the other hand, as shown in figure 22B, on 3D cell culture, Everolimus treatment diluted in low serum reduces NCI-H720 cell viability by 38% ($P < 0,01$ vs. CT) and NCI-H727 cell viability by 24% ($P < 0,01$ vs. CT). Moreover, as shown in figure 20B, in the same experimental settings, Dinaciclib treatment affects cell viability of NCI-H720 and NCI-H727 cells by 66% and 52% respectively ($P < 0,0001$ vs. CT). Differences between Everolimus and Dinaciclib treatments are significant both in 2D and in 3D. Indeed, as shown in figure 22A, in 2D culture method, Dinaciclib promotes an further cell viability reduction by 39% in both NCI-H720 and NCI-H727 cells ($P < 0,0001$ vs. Everolimus). Moreover, in 3D system, a further 28% viability reduction is induced by Dinaciclib in comparison with Everolimus in both NCI-H720 and NCI-H727 cells ($P < 0,0001$ vs. Everolimus) (figure 22B).

The same experiments were also performed diluting drugs directly in fibroblasts conditioned medium. As shown in figure 22A, drugs decrease cell viability of NCI-H720 and NCI-H727 cells also in this scenario. In particular, Everolimus decreases cell viability of 2D NCI-H720 cells by 55% ($P < 0,0001$ vs. CT) while, in the same cell line, Dinaciclib decreases this parameter by 84% ($P < 0,0001$ vs. CT). Moreover, a strong decrease in cell viability can be observed also in 2D

NCI-H727 cells. Indeed, as shown in figure 22A, Everolimus diluted in conditioned medium decreases cell viability by 33% (P < 0,0001 vs. CT) while Dinaciclib causes a cell viability reduction of 73% (P < 0,0001 vs. CT). Drugs reduce cell viability also in 3D settings. Indeed, as shown in figure 22B, Everolimus diluted in conditioned medium cause a cell viability reduction by 56% in NCI-H720 spheroids (P < 0,0001 vs. CT) and of 24% in NCI-H727 spheroids (P < 0,01 vs. CT). Moreover, as shown in figure 22B, also Dinaciclib diluted in conditioned medium cause a decrease of cell viability of both cell lines cultured in 3D. Indeed, the drug decreases cell viability by 75% (P < 0,0001 vs. CT) and 68% of NCI-H720 and NCI-H727 spheroids, respectively. Differences between Everolimus and Dinaciclib treatments performed in low serum are significant both in 2D and in 3D. Indeed, as shown in figure 22A, in 2D culture method, Dinaciclib promotes a further cell viability reduction by 29% in NCI-H720 and by 40% in NCI-H727 cells (P < 0,0001 vs. Everolimus). Moreover, in 3D system, an additional 19% and 44% viability reduction is induced by Dinaciclib in comparison with Everolimus in NCI-H720 cells (P < 0,0001 vs. Everolimus) and in NCI-H727 cells (P < 0,001 vs. Everolimus), respectively (figure 22B).

Furthermore, our results indicate that Everolimus and Dinaciclib have an enhanced activity once diluted in conditioned medium. In particular, as indicated in figure 22A, in 2D Everolimus promotes a further cell viability reduction by 15% in NCI-H720 cells (P < 0,0001 vs. Everolimus treatment in low serum) while in NCI-H727 cells influences this parameter by 10% (P < 0,05 vs. Everolimus). The same effect is observed in treatments with Dinaciclib that, in the same settings, induces a further cell viability reduction by 5% (P < 0,001 vs. Dinaciclib) in NCI-H720 cells and by 9% (P < 0,01 vs. Dinaciclib) in NCI-H727 cells (figure 22A). The same trend can be observed on cancer cells spheroids. Concerning cell viability decrease promoted by Everolimus on NCI-H720 spheroids, treatment diluted in conditioned medium increase drug effects enhancing the reduction in cell viability by 18% (P < 0,05 vs. Everolimus) (figure 22B). On the other hand, treatments in conditioned medium didn’t modify the inhibitory action of the drug on NCI-H727 spheroids.

Concerning Dinaciclib effects, the drug shows an enhanced action when treatments are performed in conditioned medium for both NCI-H720 and NCI-H727 spheroids (figure 22B). In NCI-H720 spheroids cell viability reduction increases by 10% (P < 0,05 vs. Dinaciclib) while in NCI-H727 spheroids this parameter increases by 15% (P < 0,01 vs. Dinaciclib treatment in low serum) (figure 22B).

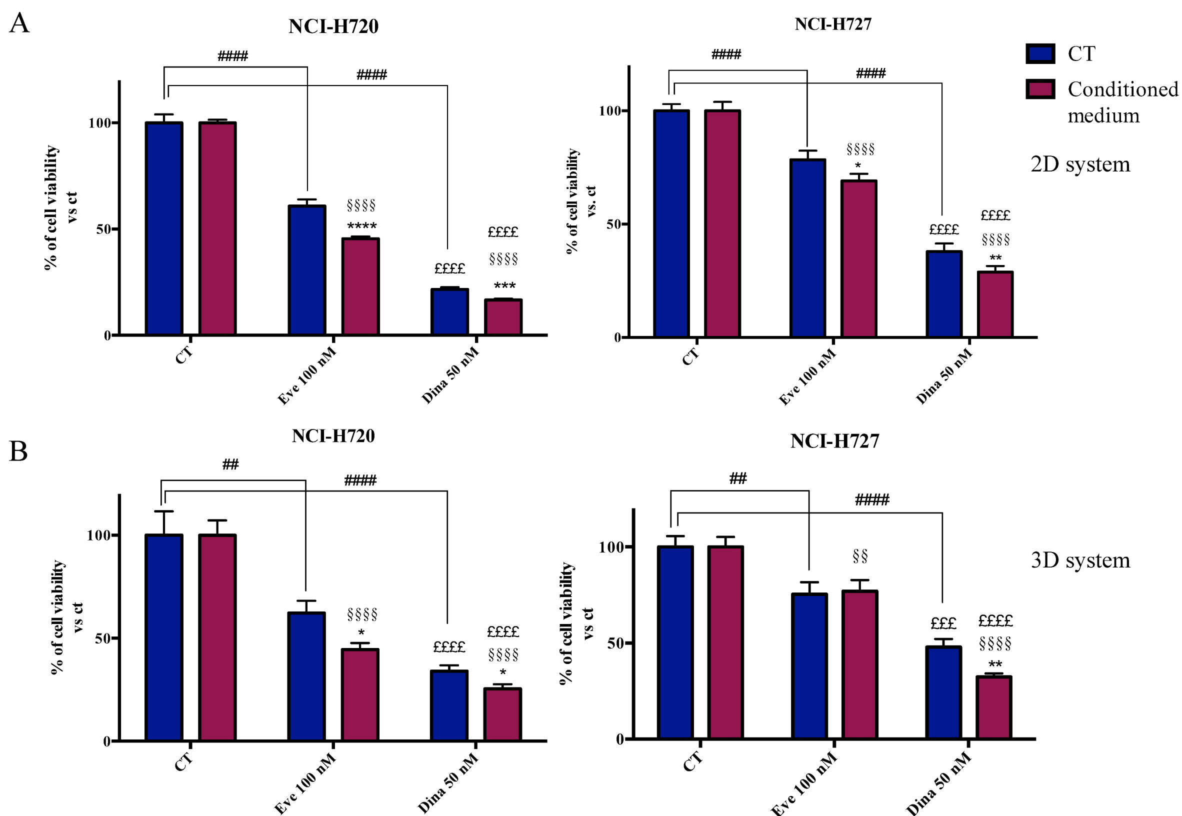

Figure 22: *Influence of Everolimus and Dinaciclib in combination with fibroblasts conditioned medium on 2D and 3D BP-NENs cells viability. BP-NENs cells were seeded in black 96 well plates for 2D culture (**A**) while for 3D culture cells were seeded in ULA plates (**B**). 2D cultures were treated the day after seeding while spheroids 72h after seeding. CT cells were incubated for 96h in low serum supplemented with Everolimus 100 nM and Dinaciclib 50 nM (blue columns) while conditioned medium cells (red columns) were incubated with Everolimus 100 nM and Dinaciclib 50 nM directly diluted in fibroblasts conditioned medium for 96h. Cell viability was measured as luminescent output in three independent experiments with six replicates each, and it is expressed as the mean ± S. E. M. £££ P < 0,001 vs. low serum Everolimus; ££££ P < 0,0001 vs. low serum/conditioned medium Everolimus; #### P < 0,0001 vs. low serum CT; ## P < 0,01 vs. low serum CT; §§§§ P < 0,0001 vs. conditioned medium CT; §§ P < 0,01 vs. conditioned medium CT; * P < 0,05 vs. low serum Everolimus or Dinaciclib; ** P < 0,01 vs. low serum Dinaciclib; *** P < 0,001 vs. low serum Dinaciclib; **** P < 0,0001 vs. low serum Everolimus. Student's unpaired T test was used for statistical analysis.*

4.3.5 INFLUENCE OF BP-NENs AND FIBROBLASTS CO-INCUBATION ON CANCERS CELLS PROTEIN EXPRESSION

In order to understand the effects of fibroblasts and their secretory factors on BP-NENs cells we have investigated cancer cells protein expression after 96h of co-incubation with fibroblasts by using transwells. We evaluated proteins commonly involved in the pathways inhibited by Everolimus and Dinaciclib and, even if the panel is not complete, the trend of proteins expression is clearly visible in figure 23. These preliminary results indicate that after 96h co-incubation with fibroblasts cancer cells start to overexpress different proteins involved in the main survival pathways that have been reported as involved in drug resistance.

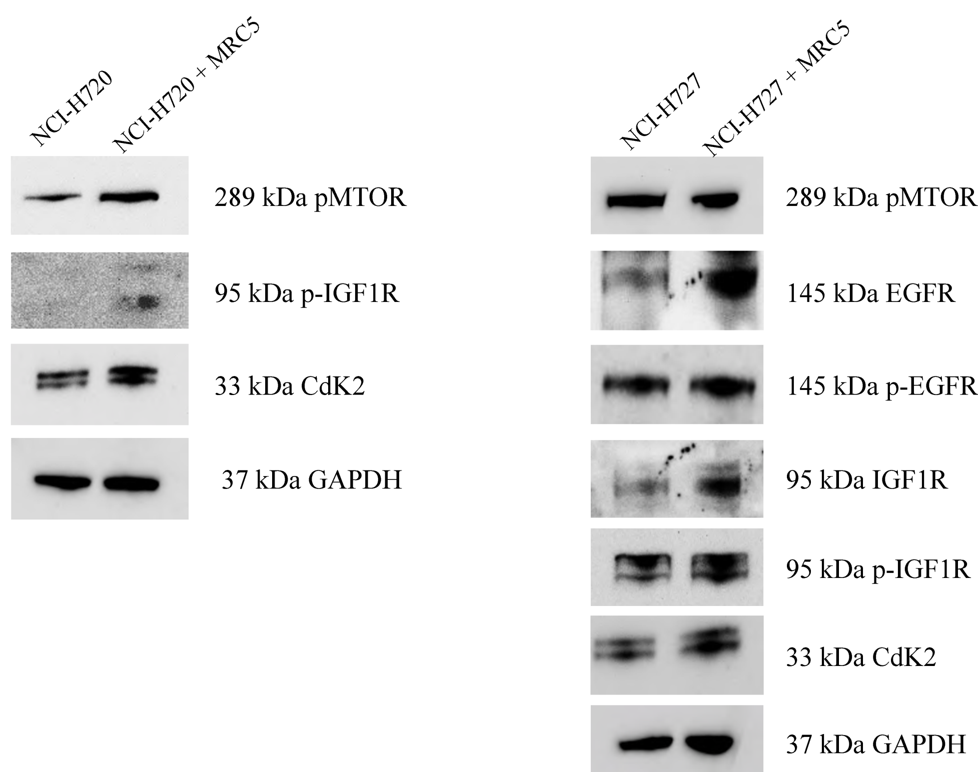

Figure 23: *Influence of BP-NENs and fibroblasts co-incubation on cancer cells protein expression. BP-NENs cells were seeded in a 6 well plate and co-cultured through the use of a transwell with MRC5 for 96h. Control cells were incubated with low serum EMEM for the same time. After cancer cells pellet collection, proteins were extracted, quantified and WB assay was performed. Membranes were incubated with primary and secondary antibodies and luminescence signal was detected though the use of Azure c300. This analysis was repeated only once. This experiment is still ongoing.*

4.3.6 INFLUENCE OF EVEROLIMUS AND DINACICLIB ON VIABILITY OF BP-NENs CELLS PREVIOUSLY CO-CULTURED WITH FIBROBLAST

Cancer cells were also incubated for 96h with fibroblasts by using a transwell, seeded again in 2D and then treated for additional 96h with Everolimus and Dinaciclib. During co-incubation with fibroblasts, control cells were incubated with no serum EMEM and complete RPMI (1:1 ratio). These experiments were performed in order to understand whether the results obtained testing drugs directly in conditioned medium could have been also observed in cancer cells pre-incubated with fibroblasts. As shown in figure 24, Everolimus decreases cell viability of both NCI-H720 and NCI-H727 cells not pre-cultured with fibroblasts by 38% and 25% ($P < 0,0001$ vs. CT), respectively. In the same settings, Dinaciclib action reduces cell viability of NCI-H720 cells by 70% while NCI-H727 cells display a reduction of 79% ($P < 0,0001$ vs. CT). On the other hand, cancer cells pre-cultured with fibroblasts show similar cell viability to the control ones. Indeed, as shown in figure 24, Everolimus decreases cell viability of NCI-H720 cells by 36% ($P < 0,0001$ vs. CT) while a cell viability reduction of 21% was observed on NCI-H727 cells ($P < 0,0001$ vs. CT). Moreover, as shown in figure 22, results obtained treating cancer cells with Dinaciclib indicate that the drug reduces cell viability of NCI-H720 cells by 70% ($P < 0,0001$ vs. CT) and by 75% in NCI-H727 cells ($P < 0,0001$ vs. CT). The comparison between the two groups, pre-incubated and not with fibroblasts, does not indicate significant differences. Also in this setting a significant difference is evident between Everolimus and Dinaciclib treatments. Indeed Dinaciclib reduces cell viability to a greater extent as compared to Everolimus (32%; $P < 0,0001$ vs. Everolimus) in NCI-H720 cells and in NCI-H727 cells (54%; $P < 0,0001$ vs. Everolimus). The same pattern is observed in cells pre-cultured with fibroblasts. Indeed, Dinaciclib is more effective in decreasing cell viability as compared to Everolimus by 34% ($P < 0,0001$ vs. Everolimus) in NCI-H720 and by 54% in NCI-H727 ($P < 0,0001$ vs. Everolimus).

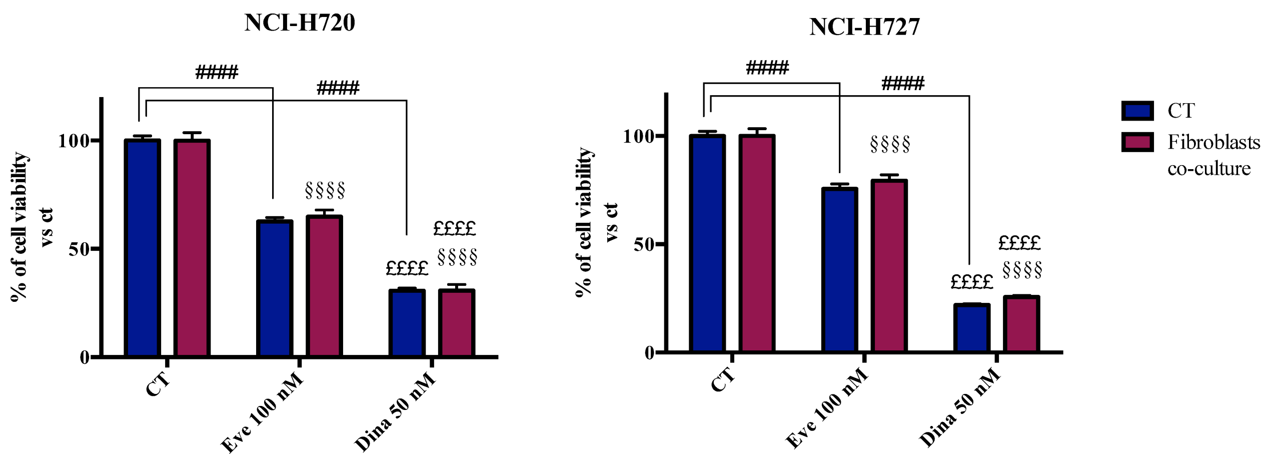

Figure 24: *Influence of Everolimus and Dinaciclib on cell viability of BP-NENs cells pre-cultured with fibroblasts. BP-NENs cells were seeded in a 6 well plate and pre-cultured through the use of a transwell with MRC5 cells for 96h (red columns). Control cells were incubated with low serum EMEM for the same time (blue columns). After 96h cells were collected and seeded again in a 2D black 96-well plate and incubated with complete RPMI supplemented with Everolimus 100 nM and Dinaciclib 50 nM. Control cells were treated with a vehicle solution. Cell viability was measured as luminescent output in three independent experiments with six replicates each, and it is expressed as the mean ± S. E. M. ££££ P < 0,0001 vs. CT/fibroblasts pre-culture treated with Everolimus; #### P < 0,0001 vs. CT; §§§§ P < 0,0001 vs. fibroblasts pre-culture CT. Student's unpaired T test was used for statistical analysis.*

5. DISCUSSION AND CONCLUSIONS

NENs are a group of neoplasms characterized by a various and fascinating range of morphologic, functional and behavioural features. This heterogeneity is mostly due to NENs origin since they can arise from several parts of the human body generating a wide family of malignancies [(2, 80)]. Each family component presents its peculiar characteristics thanks to which, during time, different classifications and clinical approaches for their management have been defined. The common feature among all the different NENs types is that the first line treatment is represented by surgical resection that, unfortunately, is not feasible in most of the cases due to metastasis occurrence [(227, 282)]. Another common feature is represented by the lack of an effective medical treatment. Several steps towards a better patients quality life have been done in the last years and different strategies and agents have been approved for the treatment of these malignancies. However, despite the effectiveness of some treatments in prolonging PFS and OS, no effective therapy has been approved [(281)]. In order to find an effective treatment for NENs a researcher can choose different approaches and, in this study, we tried to evaluate the problem under different points of view. We started from the basis, finding an effective setting to study NENs, and we continued exploring beyond already approved therapies. In conclusion, we ended with the investigation of a new approach for drug resistance comprehension. During this journey we tried to offer new prospective in NENs treatment in order to exploit all the resources already available and open innovative frontiers. In particular, during our investigation, we studied two different NENs, pNENs and BP-NENs, which represent the two main NENs in terms of incidence. In the first part, using a pNEN cell line, we evaluated the best 3D culture method in terms of easiness and reproducibility. The necessity to perform *in vitro* studies also in 3D settings has been underlined by several research groups and, therefore, in order to understand drug effects also in a more realistic system, we decided to investigate which method was the best to study NENs in 3D [(253, 255)]. Thanks to this first part we understood which way represented the best option and we applied it in the next two parts of the study. In the second

part we tried to find alternative medical approaches for BP-NENs treatment. In particular, we wanted to understand if the well-known RTKs inhibitor Sunitinib, already approved for pNENs treatment, could represent a good strategy also for the treatment of BP-NENs. This hypothesis has been postulated since this agent had already shown a good effect on BP-NENs during phase I and II clinical trials [197, 286, 287]. In addition, thanks to the evidence that EGFR and IGFR seemed to be two main Sunitinib targets in BP-NENs, we tested two inhibitors, Erlotinib and Linsitinib, EGFR and IGF1R inhibitors respectively, on BP-NENs cell lines. This choice was also supported by the need to find additional approaches besides Sunitinib, since the acquirement of resistance after a period of treatment with the agent has been already fully documented in pNENs [198, 201]. In the end, we evaluated another aspect that has already shown to be deeply correlated with tumour behaviour and drugs resistance. Indeed, we approached at the difficult topic of TME trying to understand its possible role in BP-NENs. Therefore, we evaluated how another cell type, usually present in TME, can influence response to treatments and spheroids formation. The choice to pursue this aim was supported by robust literature evidence, growing day by day, indicating that it is mandatory to stop thinking at malignancies as separate entities not included in a complex system [264, 268, 269]. In order to understand TME role in BP-NENs drugs resistance we evaluated the actions of two agents. Indeed, Everolimus, well-known mTOR inhibitor already approved for BP-NENs treatment, and Dinaciclib, multi cycline and CDKs inhibitor, were tested on cancer cells co-cultured and not with a lung fibroblast cell line.

5.1 EVALUATION OF 3D CULTURE METHODS FOR NEUROENDOCRINE NEOPLASMS STUDY

In the first part of our study we reported, to the best of our knowledge, for the first time a comparative analysis between three different 3D culture methods using a pNEN cell line. We identified ULA plates as the best scaffold-free method for reliable 3D spheroids generation. This result has been particularly important for the next two parts of the study since we have employed this culture system to obtain BP-NENs spheroids. First of all, we took in consideration a 96- well hanging drop plate as first method to culture BON1 cell line. As underlined by several research groups, this method is particularly indicated to perform drug screening/cytotoxicity assays and its most interesting feature is represented by the exploitation of cell natural tendency to aggregate without the involvement of scaffolds/matrixes [254, 290]. Among the different advantages offered by the hanging drop method, one of the most important is represented by the easiness of protocol procedures for spheroid formation. One the other hand, one of the most problematic aspect related to this method, according to our experience with BON1 cell line, was spheroids transfer after day 3. Transfer usually leads to spheroids disaggregation and this issue has been also documented by Amaral *et al*., who confirmed that one of the major weak points of the method is the need, in order to carry out cytotoxicity assays, to transfer the spheroids to a conventional 96-well plate [291]. Moreover, also other research groups reported with different cell lines that spheroids transfer can cause structural alterations [292] indicating that this issue appears to be related to the culture method rather then to the cell type. The impossibility to refresh spheroids medium, due to the small seeding volume (30 µl), represents another disadvantage related to this method. Indeed, medium cannot be replaced, since this procedure would imply the presence of a microscope under the cell culture hood in order to ensure that spheroids are not lost during medium replacement. Several research groups have documented this system peculiarity that can indeed represent an important weak point related to this method [254, 290, 293]. Moreover, find the correct focus meanwhile spheroids are located in

the hanging drop plate can represent a difficult challenge. This feature is a strong limitation that leads to the impossibility to take good quality pictures, hampering spheroids perimeter measurements. Indeed, reproducibility error was higher for the first 3D method in comparison to the third 3D method in terms of perimeter measurement. Furthermore, we performed MTT assay on BON1 spheroids treated with Sunitinib. For all the concentrations tested, observed cell viability reduction was significant with small variability indicating that MTT analysis is a good method for spheroids viability assessment.

A 24-well plate with a cell repellent surface was used as second method. The most important characteristic related to this method concerns spheroids size that, being bigger, allows to perform several assays, including IHC. Cell seeding is very easy and implicates the use only of a low attachment bottom plate and a shaker. This method allows to form spheroids with a regular round shape and, due to the flat bottom well, to take good quality pictures. Scaffold free systems that involve the use of a shaker have been reported by other research groups to be effective systems since they promote the generation of heterogeneous spheroids with optimal oxygen/nutrients gradients as well as good cell-cell contacts [(294)]. However, medium refreshment represents one of the most tricky and challenging aspects of this culture method: spheroids are difficult to locate in the well and, therefore, could be easily discarded during the procedure. This fact leads to the necessity to produce more replicates in order to reduce variability of the performed measurements and allow reproducibility. Similarly to the previous method, in order to measure perimeter variations, spheroids were treated with different Sunitinib concentrations. This method allows to detect significant differences in perimeter measurements 10 days after seeding, but may not be ideal for cytotoxic drugs assessment at high concentrations. Indeed, spheroid perimeter evaluation in these conditions was impossible since their integrity was compromised in our hands. In addition to these not encouraging feature, Herrera Martìnez et al. recently demonstrated that in BON1 spheroid obtained by using the 24-well plate with cell repellent surface spheroids size does not correlate with

DNA content [260]. Therefore, for 3D spheroid cultured with this method, perimeter analysis is not reliable as a measurement of drug cytotoxicity. Moreover, MTT evaluation is hardly achievable since spheroids should be transferred to a different smaller plate. However, the possibility to perform IHC analysis, represent the most interesting feature of this method since it allows to explore protein expression/activation directly in the different spheroid areas, helping to better understand drug mechanisms of action.

The third and last scaffold-free method involves an ULA 96-well plate. In this setting, cells proximity and, therefore, spheroids formation is promoted by centrifugation. BON1 spheroids appear very compact and with a round type morphology from the very beginning. These characteristics assume a very important meaning since have been indicated as strongly related to a 3D models key feature: a robust cell-cell adhesion [262, 295]. The formation of a single centrally located spheroid for each well is promoted by the well shape that, moreover, allow good picture quality acquisition. Spheroids generated with ULA plates, similarly to the hanging drop method, are suitable for drug testing with the difference that there is no need to transfer 3D cultures from one plate to another to perform cytotoxicity assays. Spheroids are seeded inside the plate in a 30 μl volume and treatment can be performed directly adding medium into the wells, without transferring the spheroids. The possibility to treat spheroids directly in the seeding well and, therefore, not stress/damage them with transfer, is one of the most interesting features of this method. Moreover, also in this case, spheroids perimeter was evaluated and its analysis showed significant results. Indeed, in comparison with the hanging drop method, spheroids seeded with ULA plates showed a smaller reproducibility error for perimeter measurement indicating a better reproducibility. Furthermore, ULA plates method allows MTT analysis, and results indicate a higher sensitivity in detecting viability variations as compared to the hanging drop method, probably due to a higher spheroids homogeneity.

In conclusion, we found that, when perimeter evaluation is assessed in BON1 spheroids, ULA

plates method allows to get the most reproducible results as compared to the other investigated methods. In addition, the possibility to generate a single spheroid in each well, that is not disturbed nor altered due to plate transfer/medium refreshment, allows to get better results also in following experimental stages such as viability/cytotoxicity assays.

These results are particularly important for NENs study since the lack of reliable models has hampered, during time, their comprehension and analysis.

5.2 IDENTIFICATION OF NEW PUTATIVE MOLECULAR TARGETS IN BRONCHO-PULMONARY NEUROENDOCRINE NEOPLASMS THROUGH THE USE OF 2D AND 3D CULTURES

As anticipated before, BP-NENs are a group of malignancies increasing in incidence without a specific/effective therapeutic strategy [197]. The only available molecular targeted therapy approved for the treatment of this malignancy is Everolimus, an mTOR inhibitor, which will be taken in consideration in the next part of the study. One of the most critical point concerning Everolimus treatment is the occurrence of primary or secondary resistance in patents [296]. On the other hand, for the treatment of some NENs, also other agents are approved as medical therapy. Indeed, the small multi-RTKs inhibitor Sunitinib has been approved in 2011 for pNENs treatment [188]. The interesting feature concerning this agent is that, during clinical trials, it has shown good effects also in other NENs and not only in pNENs [288, 297]. Indeed, during phase I and II clinical trials, Sunitinib has shown to produce an effect also on BP-NENs but, however, it has never been approved for their treatment [298]. The lack of data about Sunitinib action and effectiveness in NENs of extra-pancreatic origin leads to the crucial necessity to study *in vitro* the possible role of this drug on other NENs, such as BP-NENs, in order to find new strategies for the treatment of this tumour group. Therefore, through the use of two cell lines and primary cultures, we have assessed for the first time in this study the effectiveness of Sunitinib on BP-NENs. Our findings are consistent with other studies that have already demonstrated Sunitinib efficacy also in malignancies for which the drug is not approved [185, 299]. Our data suggest that EGF and IGF1 may be involved in Sunitinib mechanism of action since co-treatment with these factors counteracts, at least in part, the drug antiproliferative and pro apoptotic action. Therefore, the main Sunitinib target described in literature, VEGFR, may not represent a key regulator of its mechanism of action in BP-NENs. Only few studies have explored the possible involvement of other RTKs in drug mechanism of action, whereas our data suggest IGF1R and EGFR as key target in BP-NENs [1, 300, 301]. Moreover, in

order to better characterise the role of EGFR and IGF1R as key regulators of Sunitinib mechanism of action, we employed the ULA plates 3D culture system for spheroids generation. Through the use of this system we evaluated the impact of Sunitinib and growth factors on three-dimensional cell structure and cell viability using a staining assay. Results indicate that even in a 3D system Sunitinib has a strong antiproliferative effect that appears to be mainly focused in the centre of the spheroids. Furthermore, in order to deeply evaluate Sunitinib effects, this drug has been tested alone and in combination with EGF and IGF1 in primary cultures. Our findings indicate that treatment with Sunitinib decreases IGF1R phosphorylation levels that are partially restored with co-treatment with IGF1. Since our results indicated IGF1R and EGFR as key factors involved in Sunitinib effects modulation, we decided to better characterise the role of these two receptors in regulating BP-NENs cell lines proliferation. Therefore, we tested Linsitinib and Erlotinib, respectively IGF1R and EGFR inhibitors. Results indicate that both the agents have an antiproliferative action on the two cell lines and that a strong caspase activation is generated by the treatments. Our findings indicated also that the combination of Erlotinib and Linsitinib decrease cell viability to a greater extent as compared to single treatments and to treatment with Sunitinib. These results are mirrored by caspase activation that, particularly for NCI-H727 cells, is induced by almost three folds more as compared to treatments with Linsitinib, Erlotinib and even Sunitinib alone. Several hypotheses supported the importance of EGFR and IGF1R in NENs treatment. Indeed, Gilbert *et al*. have reported high EGFR levels in NENs samples derived from patients and that high IGF1R levels are present in BP-NEN cell lines [302, 303]. Moreover, the exploration of these agents effects on 3D spheroids structure and cell viability revealed that their action seem to act mostly in the centre of the spheroids and that Linsitinib and Erlotinib decrease cell viability with a greater extent once in combination. This observation is particularly important since underlies a synergistic effect of the two agents in BP-NENs. This synergistic effect is particularly significant for NCI-H727 since these cells have shown to be less sensitive to Everolimus and NVP- BEZ235 in a previous study and, moreover, have

shown a limited response to Sunitinib treatment as compared to NCI-H720 [277]. Therefore, in this case, EGFR and IGF1R are suggested as new potential targets useful for the development of new therapies in NENs. Moreover, results demonstrated that the block of the well-established reciprocal cross-talk between EGFR and IGF1R could be induced by the double inhibition promoted by co-treatments with Erlotinib and Linsitinib. EGFR and IGF1R relationship, in terms of adaptive activation of IGF1R family members upon the inactivation of EGFR and vice versa, has been previously demonstrated by Haluska *et al*. [304]. These authors have also showed that in ovarian and breast cancer cell lines, the co-treatment with IGF1R and EGFR inhibitors results in a synergic antiproliferative effect followed by the decrease of several important proteins involved in cancer progression, morphological changes and caspase activation in comparison to single treatments. These results support the hypothesis that more specific RTK inhibitors could produce beneficial effects and are in agreement with our findings. This can be especially significant in the management of malignancies characterised by IGF1R and EGFR overexpression such as NENs.

Finally, our results show for the first time the effectiveness of Sunitinib in BP-NENs cells and that the VEGF/VEGFR system may not be crucial in modulating BP-NENs responsiveness to the drug. Sunitinib counteraction promoted by IGF1 and EGF indicates that IGF1R and EGFR may be key regulators of Sunitinib resistance and that this resistance might be overcome using a combination of more specific IGF1R and EGFR inhibitors. In conclusion, our findings suggest that RTKs inhibition can lead to new therapeutic horizons in BP-NENs treatment and that, in order to overcome resistance, the inhibition of specific targets is required. Therefore, in order to find new molecular targets and new strategies to overcome resistance, we evaluated another potential agent for BP-NENs treatment and we tried to understand the role of a huge tumour component, the TME, in this tumour type.

5.3 NEW APPROACHES IN BRONCHO-PULMONARY NEUROENDOCRINE NEOPLASMS STUDIES, FROM THE COMPREHENSION OF TUMOUR MICROENVIRONMENT INFLUENCE ON TUMOUR BEHAVIOUR AND RESPONSE TO DRUGS, TO THE OPENING OF NEW FRONTIERES IN MEDICAL TREATMENT

Finally, after the assessment of 3D culture setting and the evaluation of Sunitinib as potential agent in BP-NENs treatment, we approached the difficult topic of TME since, interacting with tumour cells, can influence tumour behaviour, response and resistance to treatments. This approach has never been taken in consideration in BP-NENs studies for the identification of new potential markers involved in drug resistance. Therefore, this approach can represent a significant innovation for this tumour type management. TME is composed by different cell types all incorporated in a matrix outside and between cells, full of proteins and factors forming the ECM. In this complex scenario all the cell types communicate with the others generating a cross-talk able to influence their phenotype, behaviour, development and, therefore, tumour progression. Therefore, our aim was to investigate the possible connections between BP-NENs cells and TME in terms of drugs sensitivity and tumour behaviour. In order to pursue our aim, we evaluated the effects of two drugs, Everolimus and Dinaciclib, on BP-NENs cells co-cultured and not with fibroblasts. 3D cultures were essential for the generation of a complex system as similar as possible, according to our possibilities, to real tumours. Our choice was mainly based on previous literature evidence that have underlined how TME is deeply involved in NENs behaviour/resistance [264] and, since drugs resistance has represented our focus of interest in all the study, we decided to investigate its role in BP-NENs. The choice of Everolimus and Dinaciclib as treating agents was supported by previous evidence from clinical practice and *in vitro* findings in our group. Indeed, BP-NENs patients have already demonstrated to display a resistance, native or acquired, to one of the agents approved for malignancy management, Everolimus, and, consistently, previous data obtained in our group have shown that the NCI-H727 BP-NEN cell line displays a "resistant" profile to this drug [277]. In

particular, results obtained on this cell line have demonstrated that, after treatment with Everolimus, a Cyclin D1 reduction was observed however without cell cycle arrest. Therefore, thanks to these results, our interest in cell cycle progression and its druggable targets suggested to test a cell cycle inhibitor in order to further verify its possible efficacy in BP-NENs. Consequently, in this study, we have tested Dinaciclib effects on BP-NENs cells. In order to pursue our aims we generated a system enriched of two additional complexities in comparison with common 2D culture systems. Indeed, cancer cells were also cultured, through the involvement of ULA plates, in 3D and in co-culture with fibroblasts. Co-culture spheroids were generated in order to obtain a more realistic tumour representation improved under several aspects including 3D mass structure and relationships between cells. Cancer cells were seeded as spheroids alone and co-cultured and, as indicated in figure 18, NCI-H720 and NCI-H727 cells cultured alone generate two completely different spheroids. NCI-H727 cells form a compact and solid spheroid in which cells are not visible as separate entities whether NCI-H720 spheroids are not creating a proper 3D mass and cells remain visible as distinct units. Cell lines capability to generate a proper spheroid *in vitro* is not a granted feature. Indeed, as underlined also by Stadler *et al.*, cells may loose their ability to integrate into spheroids [(305)]. According to the authors the loss of this capability correlates with a lack of homotypic cell adhesion factors expression and with an increased migration and invasion capacity *in vitro*. This finding can be partially applied to our case since NCI-H720 cells naturally grow as clusters meaning that cells express important factors for 3D aggregates generation. However, according to our experience, NCI-H720 cell clusters start to be generated after 5/10 days after seeding/cell defrosting. This observation could indicate that this cell type needs cell proximity for more then 3 days, time at which cells have been observed, in order to form a proper spheroid. After this first observation we cultured cancer cells in 3D with fibroblasts addition. Moreover, in order to easily identify cell types into the sphere, we stained NCI-H720 and NCI-H727 cells with a red dye while MRC5 cells were stained with a green dye. Results indicate that tumour and fibroblast cell

lines mixed together form proper spheroids: fibroblasts seem to be mostly located in the spheroids perimeter while cancer cells seem to prefer the spheroid core. Results obtained by the mixture of NCI-H720 and MRC5 cells indicate that spheroids appear more compact, generating aggregates where single units are not visible. This observation could indicate an effect of fibroblasts on cancer cells and vice versa. As Kalluri has reported in his study [268], fibroblasts, and especially Cancer Associated Fibroblasts (CAFs), can stimulate cancer cell protein expression pattern modifications. In addition, cancer cells, after interaction with fibroblasts, can display an increased expression of proteins promoting cell-cell adhesion and cell-ECM adhesion, such as ICAM1 and VCAM1. Moreover, a recent study revealed that the heterotypic cell adhesion between cancer cells and fibroblasts facilitates morphological EMT (epithelial mesenchymal transition) of the cancer cells and, therefore, their malignant behaviour and migration [306]. Co-cultured spheroids formation was also recorded in order to show all the different steps that bring to spheroids creation with different cell types arrangements. Cancer cell location in the hypoxic spheroid core was not surprising since it has already been demonstrated that cancer cells prefer an hypoxic environment [307, 308, 309].

After system setting, we tested Everolimus and Dinaciclib on cancer cells co-cultured with or without fibroblasts in 3D. Our results indicate that the two drugs reduce cell viability of both cancer cell lines alone and in co-culture with fibroblasts. In particular, as previously demonstrated [277], Everolimus reduces NCI-H720 cell viability to a greater extent as compared to NCI-H727 cells. This reduction was observed both for cancer cells as monoculure and in co-culture with fibroblasts. On the other hand, Dinaciclib reduces cell viability of both cancer cells and cancer cells-fibroblasts spheroids by ~ 50% and it is more potent as compared to Everolimus on NCI-H727 cells. Since this cell line has shown a reduced sensitivity to Everolimus, this result is particularly important and seems to indicate Dinaciclib as a good drug for the treatment of BP-NENs. This drug has already demonstrated to be effective in several tumours, such as glioblastoma [243], and for the first time, for the best of our knowledge, we reported its efficacy also in a model of BP-NENs. Moreover, our

results indicate that NCI-H720 cells co-cultured with fibroblasts are more resistant to the inhibitory effects of Everolimus and of Dinaciclib as compared to cells cultured alone, indicating a change in cell behaviour and drug effects. Acquired drug resistance after fibroblasts stimulation has been underlined by several reports [310, 311]. An example is given by Majety *et al.* that have demonstrated how fibroblasts can influence lung cancer cell response to therapeutic agents [312]. This group has also underlined that fibroblasts influence on different cell types produces effects on several parameters, not only cell viability in 3D co-culture. This hypothesis may explain our findings in NCI-H727 cells, that do not display any evident cell viability change in response to drug treatments when co-cultured with MRC5 cells.

In order to better understand fibroblasts effects on BP-NENs cells, we collected the fibroblasts conditioned medium and we used it to treat cancer cells grown both in 2D and 3D. Results indicate that fibroblasts conditioned medium enhances NCI-H720 and NCI-H727 cell viability in 3D while in 2D this phenomenon is significant only in NCI-H727 cells. This effect has been observed in several studies and has been correlated with the presence in conditioned medium of factors, such as cytokines and growth factors, able to stimulate cell proliferation [313, 314]. The additional effect observed in 3D systems may be due to a different gene expression of cells grown as spheroids that can lead to a different protein pattern, such as differential expression of RTKs involved in regulation of cell viability [315].

Since our results indicate a positive effect of fibroblasts conditioned medium on cancer cells viability and a negative effect of Everolimus and Dinaciclib on the same parameter, we assessed drugs effects on 2D and 3D cancer cells directly diluted in conditioned medium. Data indicate that both Everolimus and Dinaciclib diluted both in conditioned medium and low serum, used as CT, decrease NCI-H720 and NCI-H727 cell viability when grown in 2D and in 3D. Also in this setting, Everolimus confirmed to be more effective in NCI-H720 as compared to NCI-H727 cells, while Dinaciclib causes a strong cell viability reduction in both cell lines cultured in 2D and in 3D.

Moreover, also treatments directly diluted in conditioned medium showed to strongly decrease cell viability of both cell lines grown in 2D and 3D. This decrease appeared to be enhanced in comparison with the one observed after treating cancer cells with drugs diluted in low serum. This phenomenon can be observed in all the tested settings, with the exception of 3D NCI-H727 cells in which Everolimus seems to produce the same cell viability reduction in low serum and conditioned medium groups. After this observation we investigated this phenomenon and we started to assess protein expression of cancer cells co-incubated or not with fibroblasts by using transwells. These experiments are still on-going and, therefore, need more replicates to be validate. As shown in figure 23, these preliminary results show an increase in expression levels of proteins involved in cell proliferation and cell cycle progression after incubation with fibroblasts. These data could be particularly important to understand the observed enhanced cell viability decrease once cancer cells are treated with drugs diluted in fibroblasts conditioned medium. Indeed, our research group in the past demonstrated in BP-NENs cell lines and in patient tissues that higher mTOR levels correlate with enhanced cell sensitivity to Everolimus [277]. Therefore, in this perspective, fibroblasts conditioned medium, rich of factors and proteins, can stimulate cancer cell proliferation and modify protein expression leading to a modulated action of molecular targeted therapies. Fibroblasts conditioned medium action on gene/protein expression pattern of cancer cells is not novel. Indeed, a different gene expression pattern after fibroblasts conditioned medium stimulation has been reported by Awaji *et al.* in pancreatic ductal adenocarcinoma. Moreover, Wang *et al.* have recently demonstrated in lung cancer that primary CAFs can modify cancer cells behaviour enhancing their invasiveness and migration [316, 317]. However, the enhanced cell sensitivity to drugs observed after treatment with conditioned medium is not visible when cancer cells are directly co-cultured with fibroblasts: in NCI-H720 cells, the effect seems to be completely the opposite. Indeed, cancer cells co-cultured with fibroblasts are less sensitive to the antiproliferative effects of the indicated drugs as compared to cancer cells not co-cultured. This observation could be explained by the fact that

cancer cells treated with drugs diluted in conditioned medium cannot generate a cross-talk with fibroblasts, but are constantly stimulated by conditioned medium factors. Indeed, as indicated by Alkasalias *et al.*, also cancer cells influence fibroblasts at different levels enhancing their transformation in CAFs and, therefore, stimulating a different fibroblasts behaviour and protein expression pattern [(318)]. This complex communication between the two cell types is completely absent when cancer cells are treated with drugs diluted in conditioned medium. In order to further investigate whether a constant fibroblasts stimulation of cancer cells is needed to generate changes in cancer cell viability we co-cultured NCI-H720 and NCI-H727 cells with fibroblasts by using transwells. Our results did not show any difference between the two groups, indicating that a constant stimulation by fibroblasts conditioned medium on cancer cells is needed, otherwise the effects are completely lost.

In conclusion, our findings indicate that TME seems to be involved in BP-NENs tumour behaviour and response to drugs. Indeed, fibroblasts have demonstrated to be important for NCI-H720 spheroid formation. Additionally, fibroblasts conditioned medium seems to stimulate cancer cells survival and appears to be related with crucial proteins expression modification. Moreover, fibroblasts seem to induce resistance to targeted therapies in one of the two analysed cell lines. These data therefore indicate the need of further studies concerning TME impact on BP-NENs for a better comprehension of cell-cell cross-talk and the identification of more effective treatments for these tumours. Finally, but not less important, our findings indicate that Dinaciclib may represent a possible treatment in order to overcome Everolimus resistance in BP-NENs. This result, observed in combination with those obtained in the pervious part of the study with Sunitinib, can indicate that, even if only few target therapies are approved for BP-NENs treatment, also other agents can be effective in their management.

5. CONCLUSIONS

In conclusion, in this study, we established a 3D culture method in order to identify new putative targets for BP-NENs treatment and investigate the possible role of TME in their behaviour and occurrence of resistance.

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