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DOTTORATO DI RICERCA IN
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TRANSLATIONAL APPROACHES TO IDENTIFY PROGNOSTIC AND PREDICTIVE BIOMARKERS IN NEUROENDOCRINE TUMORS

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1. INTRODUCTION

1.1 NEUROENDOCRINE TUMORS

Neuroendocrine tumors (NETs) consist of a heterogeneous group of neoplasms with a wide and complex spectrum of clinical behaviour, originating from cells of the diffuse neuroendocrine system (DNES) dispersed throughout the body. The first neuroendocrine tumor was described by Otto Lurbarsch in 1867 ([1]); two years later Ransom ([2]) provided the first descriptions of the classic symptoms of carcinoid syndrome. The term "*karzinoide*" was first introduced by Oberndorfer in 1907 ([3]) combined with the primary site of the tumor in order to distinguish these neoplasms, considered "carcinoma-like" because of their slow growth, from malignant adenocarcinomas. Subsequently, Gosset and Masson outlined the recognition of carcinoids as endocrine-related tumors in 1914 ([4]). The DNES cells and the tumors derived from them were discovered in the second half of the 19th century. These cells were named as *enterochromaffin cells* because of their ability to be stained using chromium salt solutions ([5]), but other definitions were used as Kulchitzky cells, deriving from the name of one of the first discoverer of these cells. Subsequently, Feyrter described "clear cells" in different organs including those with silver-reducing power (argentaffin cells) ([6]). Since these cells produced amines and peptides, a local "paracrine" function had been attributed to them and for this reason they were functionally grouped in a novel system defined as DNES. Roughly at the same period, a cancer-like epithelial neoplasm with unusual bland histology and slow-growing behaviour was identified and defined as carcinoid. The histogenetic relationship between the enterochromaffin cells and carcinoid was established once the argentaffin characteristics of some of these tumors were described ([7]). As DNES cells were able to take up precursors of biologically active amines, to produce amine products through subsequent intracellular decarboxylation and then to store them in secretory vesicles, hence APUD (amine precursor uptake and decarboxylation) was also described. APUD cells were postulated as being of neuroectodermal origin, and therefore were named *neuroendocrine cells* ([8]).

Currently, neuroendocrine cells are described by these following criteria:

- Production of neurotransmitters, neuromodulators and neuropeptides;

- Presence of dense core secretion granules from which the hormone is produced and secreted by exocytosis in response to external stimuli;
- Absence of axons and synapses.

NETs are generally slow-growing and most frequently may develop in the gastrointestinal and bronchopulmonary systems, but in pancreas, lung, ovaries, thyroid, pituitary and adrenal glands as well, reflecting the density of neuroendocrine cells in these tissues ([9]). Neuroendocrine cells, although a heterogeneous cell population, are characterized by vaso-active peptide hormones and biogenic amines secretion, which cause characteristic hormonal syndromes. Some clinical and pathologic characteristics of these tumors are specific of the organ of origin, but other aspects are shared by all neuroendocrine tumors irrespective of their anatomic site ([10]).

1.1.1 CLASSIFICATION OF NEUROENDOCRINE TUMORS

Over the past 5 decades, several proposals have appeared regarding the classification of NETs, based on embryologic origin, morphologic differences, or biochemical profile.
The first proposed classification of carcinoid tumors was based on their putative embryologic origin (foregut, midgut, or hindgut) ([11]), but since it included tumors with different morphological, functional and clinical features, it was considered unreliable. Subsequently, a histological classification was introduced; it was based on morphological characteristics, describing carcinoid tumors according to their dominant growth pattern: insular, trabecular, glandular, mixed, or undifferentiated ([12]).
In 1980, the World Health Organization (WHO) applied the term carcinoid to most of NETs. In this classification, only the endocrine tumors of the pancreas and thyroid, paragangliomas, small-cell lung carcinomas, and Merkel cell tumors of the skin were not considered as carcinoids. The latter were divided into enterochromaffin (EC cell), gastrin (G cell), and other unspecified carcinoids ([13]). Regarding the nomenclature, the term carcinoid has been repeatedly criticized because it might not adequately convey the potential malignant behaviour of many of these tumors and it was no adequate to represent the entire morphological and biological spectrum of neoplasms of the disseminated neuroendocrine cell system. Therefore, in 2000, the WHO published a classification, which was updated in 2004 and in 2010, introducing the general terms “neuroendocrine tumor” and “neuroendocrine carcinoma” ([14]). In this classification, tumors are divided into well-

differentiated neuroendocrine tumors (WDET) with benign behaviour or uncertain malignant potential; well-differentiated neuroendocrine carcinomas (WDEC), which are characterized by low-grade malignancy, and poorly differentiated neuroendocrine carcinomas/small cell carcinoma (PDEC) of high-grade malignancy (Table 1).

1a	Well-differentiated neuroendocrine tumor
1b	Well-differentiated neuroendocrine carcinoma
2	Poorly differentiated neuroendocrine carcinoma

Table 1: *WHO classification of NETs a.a 2000, adapted from Kloppel et al. ([13])*

Although this represented an important step towards defining the tumor biology of NETs, further efforts are necessary to improve the prognostic assessment of the individual NET. In consideration of this, the European Neuroendocrine Tumor Society (ENETS) introduced a proliferation-based grading system, incorporated into the 2010 WHO classification ([15]) (Table 2). In this new classification system, the terms *neuroendocrine neoplasm*, NEN, and *neuroendocrine tumor*, NET, replaced the terms *well and poorly differentiated tumors*. This classification subdivides NETs into three main categories: *neuroendocrine tumors at low grade of malignancy* named *NET-G1*, *neuroendocrine tumors at intermediate grade of malignancy NET-G2* and *neuroendocrine carcinomas or NEC,* which includes two different subtypes, *of large- or small-cell types;* these poorly differentiated carcinomas are of G3 grade. Two other categories include mixed adenoneuroendocrine carcinomas (MANECs) and hyperplastic and preneoplastic lesions.

1	Neuroendocrine tumour, NET G1 (carcinoid)
2	Neuroendocrine tumour, NET G2
3	Neuroendocrine carcinoma, NEC (small- or large-cell type)
4	Mixed adenoneuroendocrine carcinoma, MANEC
5	Hyperplastic and preneoplastic lesions

Table 2: *Classification of NETs a.a. 2010, adapted from Bosman et al. ([16])*

Mitotic rate (number of mitoses per ten high-power microscopic fields, HPF), angioinvasion and proliferative index (% of tumor cells positive by immunohistochemistry

(IHC) for the proliferation marker Ki-67, Ki-67 index) are important factors in the classification of NETs. Ki-67 is a high molecular weight nuclear protein antigen structurally associated with chromatin and thought to play a role in cellular proliferation, which regulate ribosomal expression rather than directly contributing to cell cycle progression ([17,18]). Nevertheless its expression has been noted in all phases of cellular mitosis, it is used as a surrogate marker of proliferation. Ki-67 is alternatively named MIB-1 and for its assessment the MIB1 antibody is used. Ki-67 proliferation index refers to the percentage of cells which are positive by IHC for this antigen in a tumor section ([19]). Therefore, it has been proposed to apply to gastrointestinal neuroendocrine tumors, a grading system modified from that adopted by the WHO for endocrine tumors of the lung, though referring to the proliferation status ([20]). In particular, three tumor categories are identified: G1: <2 mitoses per 2 mm^2 (10 HPF 40x magnification) and/or Ki-67 index ≤2%; G2: 2-20 mitoses per 2 mm^2 and/or Ki-67 index between 3 and 20%; G3: ≥21 mitoses per 2 mm^2 and Ki-67 > 20% (Table 3).

Grade	Mitotic count (/2 mm^2)[a]	Ki-67 index (%)[b]
G1	<2	≤2
G2	2–20	3–20
G3	>20	>20

[a]10 high-power field [HPF], 40× magnification = 2 mm^2. It is recommended to count mitoses in at least 50 fields at ×40 magnification in areas of highest mitotic density and to divide the total number of mitoses by 5
[b]MIB1 antibody; % of 500–2,000 tumour cells in areas of highest labelling

Table 3: *Grading proposal for gastrointestinal neuroendocrine tumors, adapted from Rindi et al. ([20])*

However, in order to perform an appropriate evaluation of the mitotic count, the pathological specimen must have a minimal size: indeed, 50 HPFs represent 10 mm^2. This is not feasible in a biopsy specimen where evaluation of Ki-67 is consequently required.
The histological grading into G1, G2 and G3 is performed on the basis of the proliferation fraction assessment according to ENETS scheme, firstly published in 2006. In general, G1 and G2 should refer to well-differentiated NETs displaying diffuse and intestine expression of the two general immunohistochemical neuroendocrine markers (chromogranin A and synaptophysin), while, G3 indicates a poorly differentiated neuroendocrine carcinoma; it has high mitotic counts/Ki-67 index (Ki-67 > 20%), it is often associated with fields of necrosis, and shows significantly reduced chromogranin A expression, while maintaining

intense staining for synaptophysin ([21]). In addition, another potential histochemical indicator of a higher degree of malignancy and a poorer prognosis in NETs is the p53 tumor suppressor protein ([22]). Wild type p53 plays a vital role in regulating genomic stability by controlling the cell cycle and inducing apoptosis when cell damage is irreparable. Mutations of the p53 gene are implicated in the carcinogenesis of many human cancers ([23]). In normal conditions, p53 protein is expressed in minutes and has a very short half-life; therefore its levels cannot be detected by IHC analysis. In contrast, the mutant p53 protein has a longer half-life, and usually positivity by IHC indicates a mutated form of the protein. Overexpression of p53 protein is uncommonly identified in gastro-intestinal carcinoids (GI carcinoid) ([22]). Studies of p53 positivity in GI carcinoid tumors have reported detection rates ranging from 0% ([24,25]) to 19% ([26]); proving the p53 immunoreactivity can complement histologic grading in predicting the biologic tumoral aggressiveness.

Although tumor-node-metastasis (TNM) staging systems are commonly used in the assessment of tumors, such a system was not available for NETs. Considering the clinical relevance of this system, the first TNM classification was proposed by the ENETS in 2006 (for NET of the stomach, duodenum and pancreas) and in 2007 (for NET of the ileum, colon/rectum and appendix) ([15,20]). Subsequently, first attempt to TNM classification for NETs was published in 2009 during the 7th edition of the American Joint Cancer Committee - Union Internationale Contre le Cancer (AJCC-UICC), including for the first time digestive neuroendocrine tumors ([27]). The ENETS and the AJCC-UICC had than proposed to further refine the NETs classification to include the Ki-67 scoring index and a TNM classification system ([28]). In the AJCC-UICC classification, high-grade (poorly differentiated) NECs are classified separately, by using the exocrine classification established in respective sites. When considering well-differentiated NETs, the AJCC-UICC TNM is similar to the previous ENETS/TNM proposals for intestinal anatomical sites but differs for other locations (the pancreas, stomach and appendix). As described by Rindi et al. in 2006 ([20]), the acronym TNM for NET staging proposal, is referred to:

- *T - Primary tumor:* indicates the size of the primary tumor and the degree of spread into nearby tissues (local invasion). Generally, TX means that the primary tumor cannot be assessed, T0 indicates that there is no evidence of primary tumor, Tis indicates the presence of a carcinoma in situ. T1, T2, T3, T4 describe the size and/or extent of the main tumor. The size limits indicated for T1 are those defined by the WHO for tumors with "benign behaviour" according to site-specific clinicopathological correlations ([29,14]). For T2 of the stomach and duodenum, the sizes

are those indicated for tumors of "uncertain behaviour". In the pancreas the size limit given for T2 needs to be validated ([30]). Deeply invasive tumors are included under the T3 and T4 definitions, considering site-specific features.

- *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, N1–N3 the increasing involvement of regional lymph nodes. Although the presence of regional lymph-node metastases is, per se, a negative prognostic factor in gastroenteropancreatic NETs ([31]), the prognostic significance of the number of metastatic nodes is unknown.
- *M- Distant Metastasis:* indicates the absence or presence of single or multiple metastases at any distant anatomical site (including non-regional nodes).

1.1.2 EPIDEMIOLOGY OF NEUROENDOCRINE TUMORS

Neuroendocrine tumors represent only 0.5% of all malignancies. Although these tumors have been considered rare diseases, recent epidemiologic data have revealed that their incidence has increased significantly during the last 30 years ([32]). The reasons are partially due to an increase in incidental diagnoses in patients with few or no symptoms, secondary to improved clinical awareness, widespread use of cross-sectional imaging and endoscopic techniques, as well as plasma biomarker measurement (chromogranin A) and more-accurate histopathological diagnosis ([33]). The incidence of the disease is described as the age-standardised incidence per 100,000 inhabitants per year. The Surveillance, Epidemiology and End Result database (SEER) has shown a 5-fold increase in NETs incidence (from 1.09 per 100,000 in 1973 to 5.25 per 100,000 in 2004) in the last 30 years, with no important changes in survival; moreover, the estimated incidence of 5.25 per 100,000 in 2004 is expected to reach 8 per 100,000 today ([34]). The age at presentation is related to the primary site. The appendiceal localisation is most frequently seen at young age and particularly in woman, probably due to the fact that the tumor is often found in the settings of acute appendectomy that is much more often performed in woman; excluding the appendiceal tumors, the peak incidence is at the age of 65 years ([35]). Because of NET tumor heterogeneity and nonspecific presentation symptoms, patients with NETs can suffer delays in diagnosis of up to 7 years. As a result, they often present at an advanced stage when a cure is no longer possible ([36]). The primary site of NETs is mainly in the gastrointestinal tract (61%); the lung ranks second as the source of NETs (23%) and the

remaining percentage from other organ systems. According to the US SEER database, the most common NET sites within the gastrointestinal tract are the small intestine (≈17.3%), rectum (15.9%), colon (10.9%), stomach (6%), pancreas (7%), and appendix (3.4%) ([37]). Presentation with metastatic disease accounts for 12-22%.

Considering NETs heterogeneity, overall survival is different for each tumor. Overall survival in patients who have poorly differentiated tumors and who have distant metastases is shorter than those who have well-differentiated and localized tumors. The survival has improved in the last two decades. Prognostic factors influencing survival are distant metastasis, poorly differentiated tumor, grade, age, number of liver metastasis, extrahepatic metastasis, and the presence of positive surgical margin ([38,39]). The 5-year survival is mainly associated with stage: 93% in local disease, 74% in regional disease and 19% in metastatic disease.

1.1.3 RISK FACTORS AND GENETIC TUMOR SYNDROMES

Behavioural features such as smoking ([40]) and genetic factors ([41]) have been implicated in the etiology of NETs; however, clear causative factors have not yet been delineated ([42]). NETs are sporadic in most patients, but sometimes they might be part of specific hereditary tumor syndromes such as multiple endocrine neoplasia type 1 (MEN1), multiple endocrine neoplasia type 2 (MEN2), and multiple endocrine neoplasia type 4 (MEN4). Pancreatic NETs, for example, may occur in a minority of patients with von Hippel-Lindau disease ([33,34]).

Moreover, NETs were less observed in Recklinghausen neurofibromatosis (neurofibromatosis type I) and tuberous sclerosis. Finally, other genetic syndromes such as Carney complex, non-MEN1 familial isolated hyperparathyroidism (FIHPT), Conn adenoma, characterized by single or multiple endocrine tumors, were identified and mapped over the past ten years, but the genes related to these diseases remain unknown ([43]).

- *MEN1* is an autosomal dominant disease characterised by hyperplasia and/or multiple adenomas of the parathyroid glands, single or multiple NETs of the pancreas and/or duodenum and stomach, adenomas of the anterior pituitary, NET of the thymus and lung and functioning and non-functioning hyperplasia, or adenomas of the adrenal cortex. Less common lesions associated with MEN1 include skin lesions like angiofibroma,

collagenoma, lipoma, and melanoma and peripheral or central nervous system (CNS) tumors such as ependymoma and meningioma ([44,45]). Most of the MEN1 related tumors exhibit a somatic loss of the wild type allele of MEN1 gene resulting in its inactivation. MEN1 gene is localised on chromosome 11q13 ([46]) and encode for Menin, a 67 kDa growth-suppressor protein.

- *MEN2* is an autosomal dominant disease in which RET proto-oncogene missense mutations lead to a constitutive activation of the receptor in the absence of natural ligands, predisposing to medullary thyroid carcinoma (MTC) ([41]). Three variants have been identified, MEN2A (Sipple's syndrome), in which MTC is associated to pheochromocytoma (30-50%) and primary hyperparathyroidism (10-20%); MEN2B (Gorlin's syndrome) characterized by MTC, pheochromocytoma, mucosal neuromas and skeletal abnormalities associated with a marfanoid habitus and ganglioneuromatosis of the gastrointestinal tract; the third variant of MEN2 is defined as familial MTC (FMTC), in which MTC occurs as the sole phenotype in 3 or more patients belonging to the same family ([47]).

-*MENX* is a syndrome discovered few years ago, after observing that a rat colony spontaneously developed multiple endocrine tumors, with phenotypic features shared with both MEN1 and MEN2 human syndromes, and for this reason named MENX ([48]). Genetic studies revealed that germline mutations in the Cdkn1b gene, localised at chromosome 4, which encodes CK1p27Kip1, a putative tumor suppressor gene were involved in the MENX development. $p27^{(Kip1)}$ prevent the cell cycle progression, by binding and thereby by inhibiting cyclin/cyclin-dependent kinase complexes. Particularly, Pellegata et al. found a homozygous frameshift mutation in Cdkn1b gene, resulting in a reduction in $p27^{(Kip1)}$ protein levels ([49]). Recently, it was observed that 30% of patients with MEN1–like phenotype but without MEN1 mutations, present heterozygous mutations at CDNK1B gene. The novel human MEN syndrome, associated with CDNK1B gene mutations was named MEN4, which is an autosomal dominant disorder; unlike MENX in rats is an autosomal recessive disorder ([50]).

- *von Hippel-Lindau disease (VHL)* is an autosomal dominantly inherited disease in which the most frequent tumors are retinal and central nervous system haemangioblastomas, clear cell renal carcinoma, pheochromocytoma and uni-or bilateral pancreatic lesions ([51]).

- *Neurofibromatosis type 1 (NF-1)* is the most common familial disease predisposing to peripheral nervous system tumors ([52]). The NF-1 gene is located on chromosome 17q11.2, and the protein encoded, neurofibromin, inhibits the intracellular PI3K/AKT/mTOR pathway, which has a pivotal role in regulating apoptosis mechanisms. Loss of function of the NF-1 gene results in mTOR up-regulation and tumor development ([53]).

- *Tuberous sclerosis complex (TSC):* is an autosomal dominant disorder characterised by benign hamartomas and low-grade neoplasms in multiple organs, including brain, heart, skin, kidney, lung and pancreas. The two variants, TSC1 and TSC2, are related to inactivating mutations in one of the two growth suppressor genes: *TSC1* gene is located on chromosome 9q34 and encodes for hamartin, while *TSC2* gene is located on chromosome 16p13.3 and encodes for tuberin. Mutations of these two genes result in an impaired function of the hamartin-tuberin complex, which in turn lead to the up-regulation of the PI3K/AKT/mTOR signalling pathway ([54]).

1.2 BRONCHOPULMONARY NEUROENDOCRINE TUMORS

Bronchopulmonary neuroendocrine tumors (BP-NETs) represent ~27% of all NETs and approximately 20% of all lung cancers ([55]) and comprise a heterogeneous population of tumors arising from neuroendocrine cells of the BP epithelium ([56]).

BP-NETs were thought to arise from serotonin producing Kulchitzky-type cells, also called Feyrter cells, APUD cells or enterochromaffin cells on the basis of their similarities with the intestinal Kulchitzky cells observed using electron microscopy ([57]). The bronchial Kulchitzky-type cells, currently known as pulmonary neuroendocrine cells (PNEC), are located in the basal part of the epithelium and deeper layers of the bronchial tree, resulting in tumors that may grow between the cartilaginous plates as well as endobronchially ([58]). These are the first cells to form and differentiate in the epithelium during the earliest stages of lung development, increasing in number and reaching a peak during the neonatal period; thereafter, they persist throughout life as viable population ([59]). Although their phenotypic similarities to neural cells, it is generally accepted that PNECs are of endodermal origin because they can be found in immature fetal epithelium *in vitro*, and are part of the diffuse neuroendocrine system ([60]). They usually exist as solitary cells, but sometimes aggregate to form small-innervated clusters termed neuroepithelial bodies (NEBs) ([61]).

PNECs and NEBs exhibit similar phenotypes in terms of storage of adenosine triphosphate (ATP), serotonin (5-HT) and several other amines and neuropeptides, such as gastrin releasing peptide (bombesin), calcitonin gene-related peptide (CGRP), calcitonin, enkephalin, somatostatin, cholecystokinin, and substance P (SP) in dense core vesicles ([62]). NEBs occur exclusively within intrapulmonary airways and are considered to respond to hypoxia by secretion of serotonin, inducing local vasoconstriction to decrease the bloodstream in poorly ventilated areas of the lung and thereby directing the blood towards better-ventilated areas ([63]). Solitary PNECs are located within the epithelium lining the larynx, trachea, and bronchi down to the bronchiole-alveolar junction; they are typically tall and pyramidal in shape, extending from the basal lamina of the epithelium ([59]). PNECs are characterised by apical microvilli projecting into the airway lumen, which represent the sensory part of the cells. Upon stimulation, these microvilli respond by degranulation and exocytosis of amines and neuropeptides, which exert a local paracrine and neurocrine effect on neighbouring cells and activate both extrinsic and intrinsic neurons ([62]). PNECs comprise approximately 0.4% of bronchial epithelial cells and play an important dual role, firstly as local modulators of lung growth and pulmonary differentiation during prenatal development and secondly as airway chemoreceptors during adult life ([64]).

1.2.1 CLASSIFICATION OF LUNG NETs

In 1998 Travis et al. proposed new criteria for the classification of lung NETs, in which grading and staging system were included. They were implemented in the WHO classification of 1999 and renewed in 2004 ([65]). The current WHO classification defines four histological types defined by the term “carcinoid” for low/intermediate-grade-tumors and large-or small-cell carcinoma for high-grade tumors ([66]). It is based on conventional neuroendocrine morphological features (organoid or trabecular growth pattern, peripheral palisading of the tumor cells around the periphery of tumor nests, and the formation of rosette structures) and immunohistochemical features, with different prognostic and therapeutic implications:

1) *Typical Carcinoid (TC):* defined as well-differentiated neuroendocrine tumors of low malignant potential. TCs are characterized by a classical organoid (acinar, trabecular, insular, palisading, ribbon, and rosette-like growth pattern) separated by a fibrovascular stroma. The tumor cells are polygonal with small, round, or oval

nuclei and eosinophilic, finely granular cytoplasm. Necrosis is absent and mitoses are <2/2 mm^2 ([67]). Furthermore, at electron microscopy, TCs show abundant membrane-bound secretory granules ([68]). Similar tumors having a size of <5 mm are labelled neuroendocrine tumorlets ([69]).

2) *Atypical Carcinoid (AC):* defined as well-differentiated neuroendocrine tumors of intermediate malignant potential, they are rare and often associated with regional and distant metastases. Necrosis is present and the mitotic count is 2-10/2 mm^2 ([65]). ACs are characterised by nuclear pleomorphism, hyper-chromatism, abnormal nuclear-to-cytoplasmic ratio, prominent nucleoli, and areas of increased cellularity with disorganized architecture. As compared to TC, AC has fewer granules, distributed in the cytoplasm ([68]).

3) *Large Cell Neuroendocrine Carcinoma (LCNEC):* partly resembles the organoid architecture of AC but is made of larger cells with eosinophilic and granular cytoplasm and frequent nucleoli. The cells are arranged in organoid, palisading, trabecular, or rosette-like patterns ([67]). The cells have abundant, often large patches of necrosis and the mitotic index is >10 mitoses/2 mm^2. In the 2004 WHO classification, LCNEC is classified among non-NE large-cell carcinomas, from which it should be distinguished based on the recognition of a neuroendocrine phenotype and the morphology or immunohistochemistry for neuroendocrine markers ([70]).

4) *Small Cell Lung Carcinoma (SCLC):* identified as poorly differentiated NETs of high malignant potential ([71]), represents the most common and the most aggressive lung NE neoplasm. Classically it is characterised by small cells (usually smaller than 3 small resting lymphocytes), with scant cytoplasm and condensed chromatin, with inconspicuous or absent nucleoli and a diffuse growth pattern (histologic patterns include trabeculae, spindling, nesting, palisading, rosettes, or solid-sheet-like growth, with indistinct cell borders). The mitotic rate is very high ≥11 mitoses/2 mm^2 and necrosis is frequent ([67]).

TCs and ACs are categorized together as carcinoids, TCs comprise approximately 1-2% and ACs 0.1-0.2% of pulmonary neoplasms; LCNECs comprise ~1.6-3% of all lung neuroendocrine tumors. On the contrary, the incidence of SCLC is around 13% ([61]). TCs

and SCLCs are more frequently found to be centrally located in the lung, while ACs and LCNECs more often show a peripheral localization. Furthermore, carcinoid tumors are characterised by lower Ki-67 proliferative indices compared to high-grade lung NETs ([72]).

1.2.2 BRONCHIAL CARCINOIDS

Bronchial Carcinoids (BCs) are neoplasms arising from the neuroendocrine cells of the BP epithelium. They include both Typical and Atypical carcinoids and account for ~1-3% of all primary lung tumors and ~10% of all BP-NETs ([67]). Their incidence rate ranges from 0.2 to 2 per 100,000 inhabitants per year ([73]), although it has increased over the past 30 years (6% per year), mainly due to improved detection methods and diagnostic protocols. In particular the incidence is about 0.7/100,000 in Caucasians and 0.5/100,000 in black people. BP-carcinoids are more prevalent in Asians as compared with non-Asians, whereas they are less common in Hispanic compared with non-Hispanic ([74]). The disease is slightly more common in women as compared to men ([75]) however, most of them are found accidentally. TCs represent 80% to 90% of BP-carcinoids ([76]) and occur more frequently in the fifth and sixth decades of life, even if they can occur at any age. Although they are usually referred to benign-like neoplasms, they may also show metastatic spread and behave like ACs, displaying a poorer prognosis. The SEER data indicate that LCNEC comprise ~0.3% of all lung neuroendocrine tumors, occur most frequently in the seventh decade, and are 4 times more frequent in men than in woman ([77]). SCLCs are usually diagnosed at a mean age of 60 to 70 years and comprise 9.8% of all lung tumors.
Concerning the site of BP-carcinoids, it was observed by the SEER registry (1973-2003) that right-sided lesions were the most common (59.0%), whereas 10.4% were located in the main bronchi, Figure 1.

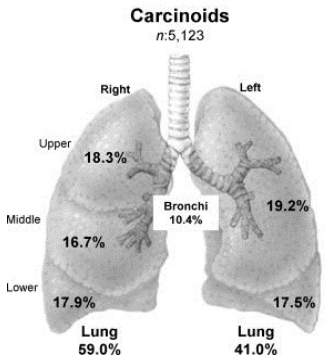

Figure 1: *Distribution of BCs, registered in the SEER registry (76). As reported by Gustafsson et al. ([123])*

Davila et al. ([78]) reported that 75% of BP-carcinoids arose in the lobar bronchi, 10% in the main stem bronchi, and 15% peripherally. The majority of TCs are centrally located, while ACs tend to be larger and are more commonly located peripherally ([79]). The etiology of BP-NETs is still unknown; differently from SCLCs and bronchial adenocarcinomas, in BCs associations with cigarette smoking, ambient radiation, or other known exposure to carcinogens are not observed ([123]). Recently, genetic risk factors have been associated with BC occurrence. Loss of heterozygosity (LOH) involving several chromosomes, such as 3p, 11q, 11q13 (MEN1 gene), 13q, 13q13 (Retinoblastoma/RB gene), 17p13 (p53 gene) has been observed in BP-NETs ([80]). High-grade lung NETs are characterised by a considerable higher number of chromosomal alterations as compared to pulmonary carcinoid tumors. Deletion of chromosome 11q13 (MEN1 tumor suppressor gene) is the only chromosomal alteration present in a considerable frequency in BC ([61]).

In contrast to high-grade lung NETs, carcinoids have a relatively favourable prognosis with a 5-year overall survival for TC tumors; recurrence occurs in only 3% to 5%, and only 15% of deaths are due to carcinoid tumors. For AC tumors, survival is clearly worse (about 70% at 5 years, 25% experience recurrence), and most deaths are due to recurrence. Almost all recurrences involve distant sites for both TC and AC ([81]).

1.3 GASTROENTEROPANCREATIC NEUROENDOCRINE TUMORS

Gastroenteropancreatic neuroendocrine tumors (GEP-NETs) also known as carcinoids and islet cell tumors are a heterogeneous group of neoplasms, with a wide and complex spectrum of clinical behaviour. They are characterised by phenotype differences and traditionally considered to originate from cells of the diffuse neuroendocrine system of the pancreas and gut ([82]). GEP-NETs are substantially rare; their incidence is about 2.5 to 5 cases per 100,000 inhabitants per year ([74]).

GEP-NETs can occur anywhere in GEP system, in general, these lesions concentrate at the gastric fundus/corpus mucosa, the proximal segment of the duodenum, the papilla of Vater, the terminal segment of the ileum, the tip of the appendix, the lower rectum and the pancreas ([83]). The cells that give rise to GEP-NETs are scattered throughout the mucosa of the gastrointestinal tract or form the islets in the pancreas described by Langerhans ([84]). They are local multipotent gastrointestinal stem cells that are able to differentiate into neuroendocrine cells, but the mechanisms underlying differentiation which involve several transcription factors (protein atonal homolog 1, neurogenin-3 and neuroD) are poorly

understood ([85]). The pancreas and the mucosa of the gastrointestinal tract contain as many as 15 cell types characterised by the ability to produce, store and secrete a variety of hormonal peptides and biogenic amines, including serotonin, somatostatin, histamine, gastrin, synaptophysin, neuron-specific enolase and chromogranin A. These secretory products are stored in large dense-core vesicles and small synaptic-like vesicles and represent morphological and functional markers of neuroendocrine cells. Secretion is regulated by G-protein-coupled receptors, ion-gated receptors and receptors with tyrosine-kinase activity ([86]). Enterochromaffin cells are the major neuroendocrine cell type of the small intestine and secrete various products in response to mechanical and chemical stimuli. The apical part of the neuroendocrine cell frequently communicates with the gut through thin cytoplasmic extension, which act as mechanosensors and chemosensors that project into the glandular lumen. These cells may show different size, shape and electron density of the secretory granules and this is important for characterising them ([87]). Enterochromaffin-like cells of the gastric fundus are part of the gastric neuroendocrine cell system and interact with antral G cells, which secrete gastrin and activate enterochromaffin-like cells to produce histamine, which in turn drives the parietal cells of the fundus to produce acid ([88]).

1.3.1 CLASSIFICATION OF GEP-NETs

Considering the biological complexity of GEP-NETs, probably due to the functional diversity and non-random distribution of the various neuroendocrine cell types from which the tumors derive, it has therefore always been difficult to assimilate GEP-NETs within a globally acceptable classification. In 1963, Williams and Sander classified the GEP-NETs according to embryological origin as foregut (stomach, duodenum, upper jejunum and pancreas), midgut (lower jejunum, ileum, appendix and caecum) and hindgut (colon and rectum) tumors ([11]). However, this classification was never generally accepted in routine diagnostic practice, because unable to distinguish the different biological relevant GEP-NETs entities. In 2000 and 2004, WHO introduced the classification for GEP-NET and for pancreatic NETs (P-NETs). As a first step, it distinguished between pure endocrine tumors and mixed endocrine–exocrine tumors. In a second step, three tumor categories were identified: well-differentiated endocrine tumors (WDETs) with probably benign behaviour, WDETs with uncertain behaviour and well-differentiated endocrine carcinomas with low-grade malignant behaviour and poorly differentiated endocrine carcinomas with high-grade

malignant behaviour. Subsequently, the well-differentiated, low-grade proliferative GEP-NETs or islet cell tumors in pancreas, were distinguished on the basis of site of origin, size, gross and/or microscopic tumor extension, angioinvasion, proliferative index (Ki- 67) and syndromic features ([89]).

In recent years, ENETS introduced guidelines for the diagnosis and treatment of GEP-NETs that contained TNM classification and grading system based on mitotic count and Ki-67 index ([15,20]). Both staging and grading system were tested for foregut and particularly for P-NETs and the biological relevance in prognosis was confirmed ([90]).

In the second half of 2010, the WHO classification was revised, introducing several changes. The NEN of the gastrointestinal tract and pancreas are stratified into two groups: the well-differentiated NETs and the poorly differentiated NECs. The NETs are then separated by their proliferative activity into either G1 (equivalent to carcinoids) or G2 NETs. The NECs, that are G3 tumors, are subtyped into small cell and large cell neoplasms. Furthermore, the neoplasms that show in addition to neuroendocrine cells (exceeding at least 30% of all tumor cells) non-endocrine components (usually adenocarcinoma structures) are distinguished from the pure NENs and called mixed adenoneuroendocrine carcinomas ([91]).

1.3.2 PANCREATIC NEUROENDOCRINE TUMORS

Pancreatic neuroendocrine tumors (P-NETs) are heterogeneous neoplasms arising from pancreatic islet cells, representing 1-4% of all pancreatic cancers, with an increasing annual incidence of 1-case/100,000 inhabitants ([92]). The trend of increasing incidence may be explained by advances in imaging, which have allowed for increased detection of incidental P-NETs (incidentalomas) ([93]). In normal conditions, enteroendocrine cells arise from pluripotent progenitor cells localised in the pancreatic ductal epithelium, which differentiate into the various hormone-producing cells of the islet of Langerhans. P-NETs are thought to arise from neoplastic neuroendocrine cells that differentiate but retain the pluri-hormonal capabilities of the progenitor cells ([94]). P-NETs can occur at any age, however, most present during the 4^{th} to 6^{th} decades, and no gender predilection has been demonstrated. Although the majority of cases are sporadic, 10-30% have been shown to be associated with MEN1 syndrome, and <1% with von Hippel-Lindau disease. Other genetic syndromes in which P-NETs may present include neurofibromatosis type 1 and tuberous sclerosis ([95,96]). P-NETs are commonly discovered incidentally, with abdominal or back

pain, anorexia-cachexia, weight loss, and peptide-specific functional syndromes representing common symptomatic clinical presentations ([97]). P-NETs can be clinically classified as *functioning* and *non-functioning*, the latter being far more common and typically presenting late during disease evolution ([98]). Functioning P-NETs are associated with clinical syndromes caused by inappropriate secretion of hormones. This group includes insulinomas, gastrinomas, glucagonomas, vasoactive intestinal peptideomas (VIPomas), somatostatinomas and some less common cancers. Clinical symptoms of these various tumors are unfortunately nonspecific, commonly resulting in delayed diagnosis ([99]).

- *INSULINOMA* is the most common form of functioning P-NET, accounting for 70-80% of cases and are typically hypervascular and predominantly located in the body and the tail of pancreas. 90% are benign, solitary and sporadic. However, 5-10% of them are associated with MEN1; among MEN1, patients will develop an insulinoma by the age of 40 years. The disease in these cases is often multifocal and malignant in up to 25% ([100]). Tumors tend to be small in size (<2 cm) at the time of presentation and without mass effects ([101]). Insulinomas can occur at any age, but mainly in middle age and show a female preponderance. They commonly present with hypersecretion of insulin and the subsequent development of symptoms of neuroglycopaenia (headache, lethargy, dizziness, diplopia, blurred vision, amnesia and more rarely seizures, coma or permanent deficits), and symptoms resulting from the catecholaminic response (tremor, anxiety, palpitations, nausea, hunger and sweating) ([102]).
In 2009, the Endocrine Society published evidence-based clinical practice guidelines for the evaluation and management of hypoglicemia. True hypoglycemia should be suspected when Whipple's triad is fulfilled: adrenergic and neuroglycopenic symptoms of hypoglycemia, simultaneous blood glucose below 55mg/dL, and relief of symptoms with administration of carbohydrate and correction of the hypoglycemia ([103]). Biochemical diagnosis of insulinoma requires demonstration of inappropriately elevated insulin, C-peptide, and proinsulin levels in the presence of low serum glucose. Once the biochemical diagnosis of insulinoma is secure, the tumor is localised radiologically.

- *GASTRINOMA* is a gastrin-secreting tumor derived from either the duodenum or the pancreas, causing the Zollinger-Ellison syndrome (ZES) by hypersecreting gastrin, which results in hyperchlorydria and gastric mucosal thickening shown as hypertrophy on endoscopy and imaging ([104]).

Two-thirds of cases are sporadic with the remaining one-third associated with MEN-1 in up to 25%; MEN-1 related gastrinomas are usually small, located in the duodenum and frequently multifocal ([105]). The majority of gastrinomas are malignant (50-85%) and up to one-third of patients present with liver and bone metastases ([106]). Gastrinomas distinguish themselves from the other P-NETs by their predilection for the "gastrinoma triangle" that includes the pancreatic head, the first two-thirds of the duodenum, and the porta hepatic. The diagnosis of gastrinoma is made when serum gastrin levels are inappropriately elevated in the setting of excessive gastric acid production.

- *GLUCAGONOMA* is rare slow-growing tumor arising from the pancreatic α-cells, commonly associated with a characteristic syndrome resulting of excessive secretion of glucagone and other peptides ([107]). The majority are sporadic, but between 5 and 17% are associated with MEN-1. Glucagonomas usually present at the 5th decade of life, they are highly malignant (75%) and mainly located in the tail of the pancreas; the sporadic tumors mainly present hepatic metastases at diagnosis. The most common symptoms are weight loss, diabetes mellitus, cheliosis or stomatitis and diarrhoea; the most characteristic of these symptoms is the rush, necrolytic migratory erythema (NME) ([105]). The diagnosis is usually assured when serum glucagon level is 500-1,000 pg/ml (normal 50-150 pg/ml) even if there are many clinical settings in which serum glucagon is secondarily elevated, such as in cases of sepsis, celiac disease, hypoglycaemia, Cushing's syndrome, diabetic ketoacidosis. Furthermore, glucagon can also be released by other types of islet cell tumors ([107]).

- *VASOACTIVE INTESTINAL PEPTIDEOMAS (VIPomas)* are rare sporadic islet-cell tumors presenting in the 4th to 5th decade of life and approximately 70-80% originate from the pancreatic tails. Primary tumors are usually large, greater than 2 cm, and 50-60% of them have already developed metastases at the time of diagnosis. VIPomas can be multifocal in 4% and present with the clinical syndrome of watery diarrhea, hypokalemia, and achlorydria also known as the Verner-Morrison syndrome or pancreatic cholera. Biochemical diagnosis is made when a marked elevation (>200 pg/dL) in the serum level of vasoactive intestinal peptide (VIP) is found. VIPomas in up to 1-5% of cases can be associated with MEN-1, as well as with other neuroendocrine tumors such as pheochromocytoma and ganglioneuroma ([108]).

- *SOMATOSTATINOMA* is an islet cell tumor that may occur in both the pancreas and the duodenum and is usually malignant ([109]). It can present with symptoms of excessive somatostatin activity called "somatostatin syndrome", that is based on the inhibitory effect of somatostatin on the secretions of endocrine and exocrine glands of the gastrointestinal tract. These effects may result in diabetes, cholelithiasis, achlorhydria, and steatorrhea. Given the nonspecific nature of these symptoms, the tumors are detected when tumor burden results in a mass effect or metastases. Somatostatinomas can occur sporadically or in association with MEN-1 ([100]). The diagnosis is confirmed by the finding of marked elevations of serum somatostatin by radioimmunoassay.

Non-functioning P-NETs (or inactive, clinically silent) account for 25% to the majority of P-NETs ([110]). They are not associated with clinical syndromes caused by hormonal hypersecretion ([100]), but they may in many cases release peptides that can be detected in the serum, which are chromogranin A, pancreatic polypeptide, pancreastatin and human chorionic gonadotropin. Non-functioning P-NETs are most often diagnosed in the 5th to 6th decade of life, either incidentally or by symptoms caused by an expanding mass (obstructive jaundice, abdominal pain, palpable mass) or metastases (weight loss, haemorrhage, enlargement of the liver) as approximately two-thirds are truly malignant ([111]). The majority of non-functioning P-NETs are unifocal except when associated with MEN-1 (20-30%), where multiple tumors are generally found throughout the pancreas.

1.4 DIAGNOSIS OF NEUROENDOCRINE TUMORS

The clinical presentation of neuroendocrine tumors varies according to the site and size of the primary tumors, the presence or absence of metastatic spread, whether associated features compatible with a hereditary syndromes or not, whether the tumor is functional or non functional, and if so, what syndrome is present ([82]). Early on in the disease process, patients present with disparate symptoms associated with various hormonal syndromes (if present) that are often misdiagnosed for many years. In such a situation, early diagnosis depends on syndromic recognition and it is achieved by appropriate laboratory testing later followed by imaging studies and a tissue diagnosis ([113]). Non-functional tumors commonly present late in the disease course with metastases often identified on imaging or when studies are ordered for symptoms attributable to tumor growth rather than hormone production. In these cases, imaging studies (often followed by a tissue diagnosis)

commonly precede biochemical testing. Therefore, the initial diagnostic approach in patients with NETs includes histological examination, which is always required before therapeutic decisions are made. Clinicians should also consider performing repetitive biopsies to reassess the prognosis if the disease course changes significantly. The following investigations are also required: (a) immunohistochemical markers and detailed histological analysis; (b) assessment of the primary tumor and the extent of extrahepatic spread by imaging, including patterns of hepatic metastases; and (c) biochemical assessment of functionality and general tumor markers ([112]).

1.4.1 CIRCULATING BIOMARKERS

Neuroendocrine tumors derive from neuroendocrine cells, which secrete peptides and biogenic amines throughout the paracellular environment and the systemic circulation. These products are tumor specific and may be useful as markers for the diagnosis and follow-up of treatment. Some tumor markers may have prognostic implications ([113]). These include serotonin (5-HT), histamine, gastrin, tachykinins and somatostatin. The amine and peptide producing cells utilise endocrine, paracrine, neurocrine or autocrine regulatory mechanisms ([114]). The secretory pathway is an intricate process that comprises multiple, tightly regulated steps. After their synthesis in the endoplasmic reticulum, hormones and neuropeptides are sorted and packed into large dense core vesicles (also named secretory granules) in the Golgi apparatus. Therefore, the cytoplasm of the neuroendocrine cell is occupied by a large number of secretory granules of varying electron densities, size and shape (100-400 nm in diameter) and is the storage site of secretory products. Upon specific stimulation, granules are translocated to the cell membrane in a cytoskeleton-dependent manner and mature into competent organelles for secretagogue-induced exocytosis. Granules are then tethered to the plasma membrane, docked, and primed, before finally releasing their contents by exocytosis mediated by G-protein-coupled receptors, ion-gated receptors, and receptors with tyrosine-kinase activity after fusing with the plasma membrane ([115]). Activating pathways for secretion of bioactive products include adenylyl cyclase, β-adrenoreceptors, and pituitary adenylate cyclase-activating polypeptide, whereas somatostatin (via somatostatin receptor 2), acetylcholine (via muscarinic M4 receptors), and gamma-aminobutyric acid (via gamma-aminobutyric acid A receptors) inhibit secretion ([116]). Peptide pro-hormones are synthesised in the rough endoplasmic reticulum (RER), together with chromogranin A (CgA) and other granular proteins. Chromogranins

act as substrates for proteolytic enzymes and thereby modulate this process ([114]). The secretory products are divided into general markers and specific markers, depending on the cell type involved. General tumor markers such as chromogranin A, pancreatic polypeptide, serum neuron-specific enolase and subunits of glycoprotein hormones have been used for screening purposes in patients without distinct clinical hormone-related symptoms ([117]).

Chromogranins: constitute a whole family of glycoproteins of which chromogranin A (CgA) and B (CgB) are the most clinically interesting ([118]). These proteins contain 10% acidic (glutamic or aspartic acid) residues, as well as single or multiple dibasic amino acid residues ([119]). They are found in neuroendocrine cells throughout the body, but are also located in the neuronal cells in the central and peripheral nervous systems ([120]). CgA is a 49 KDa acidic glycoprotein expressed in the secretory granules of most normal and neoplastic neuroendocrine cell types; it contains multiple pairs of basic amino acids distributed along its length, but more abundantly in the carboxyl terminal part of the molecule. CgB has a similar chemical structure. CgA has proven particularly useful for monitoring disease response and progression in patients with carcinoid tumors ([121]). NETs usually present with increased plasma levels of CgA and sometimes also CgB. Elevated circulating CgA levels have been detected in serum or plasma of patients with various NETs including phaeochromocytomas, paragangliomas, pancreatic islet cell tumors, medullary thyroid carcinoma, small intestinal NETs, parathyroid and pituitary adenomas, and also in a proportion of patients with small-cell lung cancer. The highest CgA levels have been found in patients with metastatic carcinoids and islet cell tumors ([122,123,124,125]). CgA have demonstrated a sensitivity that varies between 53% and 68% and specificity between 84% and 98% ([36]). The primary structure of human CgA contains 10 pairs of basic amino acids, which are potential cleavage sites for specific endogenous proteases, which provide biologically active fragments such as vasostatins, pancreastatin and chromostatin. Many of the biological effects attributed to CgA seem to be mediated by these peptides ([113]).

Pancreatic Polypeptide (PP): PP is 36-amino acid linear peptide secreted by pancreatic polypeptide cells, which are located in the gut mucosa and pancreas. It has been found to be elevated in NETs of the gastrointestinal tract and pancreas, with a sensitivity of about 50–80% ([126]). A combination of CgA and PP has been useful in patients with non-functional pancreatic neuroendocrine tumors (P-NETs), with a sensitivity of almost 95%.

Neuron Specific enolase (NSE): is the neuron-specific isomer of the glycolytic enzyme 2 phospho-D-glycerate hydroxylase or enolase. It is mainly present in the cytoplasm of cells of neuronal and neuroectodermal origin and can serve as a circulating marker for NETs. Patients with small-cell lung cancer show high NSE expression levels, but it has also been found to be elevated in 30-50% of patients with intestinal NETs, medullary thyroid carcinoma, P-NETs and pheochromocytoma, especially the poorly differentiated ones ([127]). Increased levels of NSE are also correlated with tumor size, although the specificity is lower than that of CgA. Despite its high sensitivity (100%), its use is limited as a blood biochemical marker for NETs because of its very low specificity (32.9%) ([128]). The combination of CgA and NSE has a higher sensitivity than either parameter separately ([120]).

Human Chorionic Gonadotropin (hCG): is a glycoprotein hormone consisting of alpha and beta subunits that can be ectopically produced by neoplasms. hCG alpha and beta subunits have been used as markers to screen for a number of different tumors including NETs. In particular, high hCG alpha and beta subunits levels have been found in patients with malignant P-NETs ([129]).

5-hydroxyindole-3 acetic acid (5-HIAA) is a serotonin metabolite excreted in urine that can be used to identify certain types of functioning NETs for example, distal ileum or proximal NET ([130]). An elevation in urinary 5-HIAA excretions over 24 h provides a specificity of about 90% ([131]). In addition to general markers, there are specific biomarkers for the different functioning NETs (Table 4).

Site	Tumor Type	Marker	Specificity
All		CgA and CgB	High
		PP, NSE, neurokinin, neurotensin	Intermediate
		HCG-α and HCG-β	Low
Thymus	Foregut carcinoid	ACTH	Intermediate
Bronchus	Foregut carcinoid, small-cell lung carcinoma	ACTH, ADH, serotonin, 5-HIAA, Histamine, GRP, GHRH, VIP, PTHrp	Intermediate Low
Stomach	Foregut carcinoid, gastrinoma, ghrelinoma	Histamine, gastrin Ghrelin	Intermediate Low
Pancreas	Gastrinoma, insulinoma, glucagonoma	Gastrin, insulin, proinsulin, glucagon, somatostatin	High
	Somatostatinoma, PPoma, VIPoma	C-peptide, neurotensin, VIP, PTHrp, calcitonin	Low
Duodenum	Gastrinoma, somatostatinoma	Somatostatin, gastrin	High
Ileum	Midgut carcinoid	Serotonin, 5-HIAA	High
		NKA, neuropeptide K, SP	Intermediate
Colon and rectum	Hindgut carcinoid	Peptide YY, somatostatin	Intermediate
Bone	Metastasis	bAP, N-telopeptide	High (blastic lesions), modest (lytic lesions)
		Vitamin D25, 1:25-OHD	Universal vitamin D deficiency
		PTH, PTHrp	Intermediate
Cardiac involvement	Carcinoid	BNP	Intermediate

Shows the specific biochemical markers used for each tumor and their specificity.
1:25-OHD indicates 25-hydroxyvitamin D; ADH, antidiuretic hormone; GRP, gastrin-releasing peptide; HCG, human chorionic gonadotropin.

Table 4: *Specific Biochemical Markers for Each Tumor Type*

As previously described, carcinoid tumors have been divided according to the embryological origin of the precursor cells into foregut (lung, thymus, stomach and duodenum), midgut (jejunum, ileum, appendix and caecum) and hindgut (distal colon and rectum) carcinoids and together with P-NETs, collectively considered as gastroentero-pancreatic neuroendocrine tumors (GEP-NET) ([132]). Specific markers for various types of NETs include both structural and functional products, which form the basis of another classification system. Besides this classification, NETs are also divided in functional tumors that produce a clinical hormone-related syndrome, versus non-functional tumors, which are clinically silent and might present symptoms related to tumor growth ([123,126]).
Furthermore, new potential biomarkers have recently been identified for NETs diagnosis and therapeutic tools. Particularly, a class of natural occurring small non-coding RNA molecules, the so-called micro-RNA (miRNAs) have been correlated with intestinal NETs tumor progression ([133]). MicroRNA-133a was found down-expressed in lymph node and liver metastases as compared to primary tumor and normal enterochromaffin-like cells, suggesting that its down-regulation might be related to tumor progression ([134]). Recently, a Circulating Neuroendocrine Gene Transcript Analysis (NETest) has been proposed to assess NET biological activity. The NETest measures the expression of NET marker genes in peripheral blood using gene inference technology ([135]). It has demonstrated clinical utility for the diagnosis, identification of residual disease and disease progression.

Particularly, the NET gene blood analysis has demonstrated efficacy as diagnostic and prognostic marker in well-differentiated paragangliomas and pheochromocytomas ([136]). It was further observed that the measurement of circulating NET transcripts could define the therapeutic efficacy of peptide receptor radionuclide therapy (PRRT) in neuroendocrine tumors ([137]), and predict responses to somatostatin analogs (SSAs) in GEP-NETs ([138]).

1.4.2 DIAGNOSTIC IMAGING

A wide variety of diagnostic methods are available to localize NETs. Localisation procedures able to identify both primary and metastatic tumors can be divided into three categories: cross-sectional imaging techniques, functional imaging and endoscopic approaches. The most commonly carried out cross-sectional imaging studies include computed tomography (CT) scan, and magnetic resonance imaging (MRI). Both can be used to locate primaries and to evaluate metastatic disease but are not able to distinguish functionality. Triphasic computed tomography is indicated in the initial diagnosis and follow-up evaluation of primary tumors and their distant metastases ([139]). MRI technology 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 ([140]). Somatostatin-receptor scintigraphy (SRS) is a functional imaging method that measures the binding of radiolabeled somatostatin analogs to somatostatin receptors on the surface of NETs. Somatostatin receptors are G- protein coupled membrane glycoproteins. To date, five subtypes have been identified, that are highly expressed in NETs. The somatostatin analogs octreotide and lanreotide bind with high affinity to receptor 2 and 5 such that radioactively labelled somatostatin analogues allow for the visualisation and staging of tumors expressing these receptors subtypes ([141]). The most commonly used radioligand for SRS is (^{111}In)DTPA-octreotide (indium 111-In pentetreotide, generally referred to as OcreoScan). SRS showed a reported sensitivity of ~90% and specificity of 80% ([131]). SRS is recommended at the initial diagnosis for all patients with suspected NETs ([142]) but its role in disease follow-up assessment and surveillance is not clearly established. Another radioisotope uptake method uses ^{123}I-metaiodobenzylguanidine (^{123}I-MIBG), which is actively taken into NET cells by way of norepinephrine transporters and stored in neurosecretory granules, to identify patients with inoperable or metastatic disease who might be candidates for targeted radiotherapy. The latter method is used in only a limited group of patients with small intestinal NETs ([143])

Positron emission technology (PET) is used in the diagnosis of poorly differentiated disease or well-differentiated disease with high proliferation rates or rapid clinical progression. The presence of somatostatin receptors on NETs has been exploited in PET with (^{68}Ga) Octreotide. (^{68}Ga) Octreotide/Octreotate, that has higher sensitivity and specificity for NETs than traditional somatostatin receptor scintigraphy. In addition, (^{18}F)L-DOPA has been shown to visualise carcinoid tumors with a sensitivity of 67%. Furthermore, since (^{11}C)hydroxytryptophan ([^{11}C]HTP) is taken up by carcinoid tumor cells, decarboxylated and then stored in vesicles as (^{11}C)5-HT, PET scanning with (^{11}C)5-HT can visualise carcinoid tumors that lack type 2 somatostatin receptors and are negative in SRS ([144]). Endoscopic ultrasound (EUS) is highly sensitive for determining the site and depth of invasion, facilitates the guidance of fine-needle aspiration, and is especially useful for P-NETs. Distinguishing functionality of the tumors based upon its ultrasonic characteristics has not been possible.

1.5 MANAGEMENT OF NEUROENDOCRINE TUMORS

1.5.1 SURGERY AND CHEMOTHERAPY

The heterogeneous nature of NETs, their presentation at a relatively advanced stage, and the diverse type of symptoms contributes to a wide range of treatment options. Surgery to remove the primary malignancy (complete resection of the tumor) and/or local lymph nodes (if affected) is currently the only possible cure for patients with NETs. Surgery with curative intent represents the traditional first-line strategy for treating patients with localised tumors or NETs showing only regional spread ([145]). However, because half of patients with NETs present with metastatic disease at the time of diagnosis, curative surgery is often not feasible. In these patients, palliative surgery to remove or debulk the primary tumor is recommended, but depends on its location and involvement of surrounding tissues. At least 90% resection of NETs is required to achieve symptom control, which may decrease the secretion of bioactive substances and also increase the systemic therapy's efficacy ([146]). Patients with liver metastases have a poorer prognosis than patients without metastases; in these cases, embolization or chemoembolization may be used to provide a good symptom relief. In addition, focal ablative therapies, such as radiofrequency ablation, can be effective in achieving local control of liver metastases

([147]). In addition to surgery and local ablation therapy, patients with metastatic NETs should also receive systemic therapy to control hormonal symptoms and limit tumor progression. The use of single chemotherapeutic agents (doxorubicin, 5-fluorouracil, dacarbazine, cisplatin, carboplatin, etoposide, streptozocin) has been evaluated in patients with NETs, but showed little beneficial effects in reducing the tumor mass or in controlling symptoms. Therefore, combined chemotherapy is used to improve the efficacy; in patients with P-NETs, streptozocin in combination with other agents, such as 5-fluorouracil, cisplatin or doxorubicin, has given promising response rate of about 40% ([148]). Another chemotherapeutic agent, temozolomide, has shown promising antitumor effects in patients with P-NETs; this agent is most effective in tumors with low levels of the DNA repair enzyme MGMT; and P-NETs are often MGMT deficient ([149]).

In general, chemotherapy should be considered for those patients with more rapidly progressing tumors or those who have progressed on less toxic treatments ([150]); however, additional prospective, randomized studies are needed because treatment principles generally are extrapolated from the experience with the more common NETs.

1.5.2 SOMATOSTATIN ANALOGUES (SSA)

Secretory cells express somatostatin receptors on the cellular surface that are occupied by somatostatin, an endogenous inhibitor of various hormones secreted by the endocrine system, including serotonin, insulin, glucagon and gastrin. Somatostatin binds with high affinity to the five somatostatin subtypes ($sstr_{1-5}$), leading to different inhibitory effects on the body. Each type of NET (insulinoma, gastrinoma, VIPoma, glucagonoma) can express more than one subtype, and the frequency of their expression on NETs is highest for $sstr_2$, followed by $sstr_1$, $sstr_5$, $sstr_3$ and $sstr_4$ ([151]). $Sstr_2$ and $sstr_5$ mediate antisecretory effects of somatostatin and somatostatin analogues by inhibiting hormonal secretion in functioning NETs. Some evidence suggests that somatostatin receptors mediate antitumor effects of somatostatin and somatostatin analogues through the arrest of cell growth, the extent of which depends on receptor selectivity ([152]). Somatostatin has limited clinical utility due to its short half-life (<3 min), and thus, synthetic somatostatin analogues have been developed as somatostatin receptor agonists to block hormone release. Octreotide (Sandostatin®) was the first somatostatin analogue commercially available with high affinity for $sstr_2$ and moderate affinity for $sstr_3$ and $sstr_5$. It probably exerts its antitumor effects stimulating $sstr_2$, which mediates cell-cycle arrest and apoptosis. Additionally,

Octreotide may inhibit the anti-apoptotic hormone insulin-like growth factor 1, growth factors and trophic hormone secretion, and angiogenesis ([153]). Lanreotide (Somatuline® Depot) is another long-lasting somatostatin analogue with a similar binding profile of Octreotide. Both these agents can be used to control clinical symptoms caused by hormonal secretions in NETs that predominantly express $sstr_2$ and $sstr_5$. To date, the most effective formulations include lanreotide autogel (60 mg, 90 mg, or 120 mg) and long-acting octreotide (10 mg, 20 mg, or 30 mg), which are widely accepted as effective in controlling tumor-related symptoms in about 75% of patients and in reducing serum concentration of tumor markers. These drugs are well tolerated and safe, with mild adverse effects and high tolerability after sustained use ([154]). Pasireotide (SOM230) is a novel multireceptor-targeted analogue with high affinity for $sstr_1$, $sstr_2$, $sstr_3$, and $sstr_5$. Because of its broad receptor binding profile, Pasireotide may benefit a wider spectrum of patients with hormonal symptoms of NETs who have not responded to treatment with Octreotide and Lanreotide ([155]).

1.5.3 RADIONUCLIDE THERAPY

Another therapeutic option for patients, whose tumors continue to grow after treatment with somatostatin analogues or interferon, is represented by radiation therapy that generally is used in cases with inoperable tumors or as an adjunct when a resection is incomplete. Systemic PRRT is based on delivering a radionuclide coupled to a somatostatin analogue to cells expressing somatostatin receptors and it is considered a treatment option for symptomatic patients with non-resectable metastases ([143]). Although the response of NETs to radiation is limited, PRRT has shown potential beneficial effects in patients with unresectable somatostatin-positive NETs. Different radionuclides such as ^{111}Indium, ^{90}Yttrium, ^{177}Lutetium are used linked to an SST analogue allowing to target the SST receptor"over-expressing" tumor cells. DOTA^0-Tyr^{3}octreotate is considered the most effective agent, producing tumor remission in ~50% of patients ([156]). The median duration of the therapy response in carcinoids for ^{90}Y-DOTA^0-Tyr^{3}octreotide and ^{177}Lu-DOTA^0-Tyr^{3}octreotate is 30 months and more than 36 months, respectively ([157]). Radiolabeled somatostatin analogs (^{111}In-octreotide and ^{111}In-lanreotide) are used in Somatostatin Receptor Scintigrafy (SRS) in order to measure analogue uptake and predict therapeutic efficacy. PRRT is associated with disease stabilization in ~50-70% of patients at 1-2 year follow-up ([33]).

1.5.4 NOVEL MOLECULARLY-TARGETED THERAPY

In recent years, a strong interest in identifying molecular alterations in NETs has been developed, that may point to new potential pathways and therapeutic targets. NETs demonstrate increased expression of numerous growth factors and their receptors, including vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), platelet- derived growth factor-α (PDGFR-α), platelet-derived growth factor-ß (PDGFR-ß), insulin-like growth factor 1 (IGF-1), transforming growth factor alpha and beta (TGF-α and TGF-ß), mammalian target of rapamycin (mTOR), basic fibroblast growth factor (bFGF), and stem cell factor (c-kit) ([158]). Novel agents targeting the VEGF pathway, as well as mTOR, a downstream mediator of several signalling pathways, have shown particular promise in NETs (Figure 2).

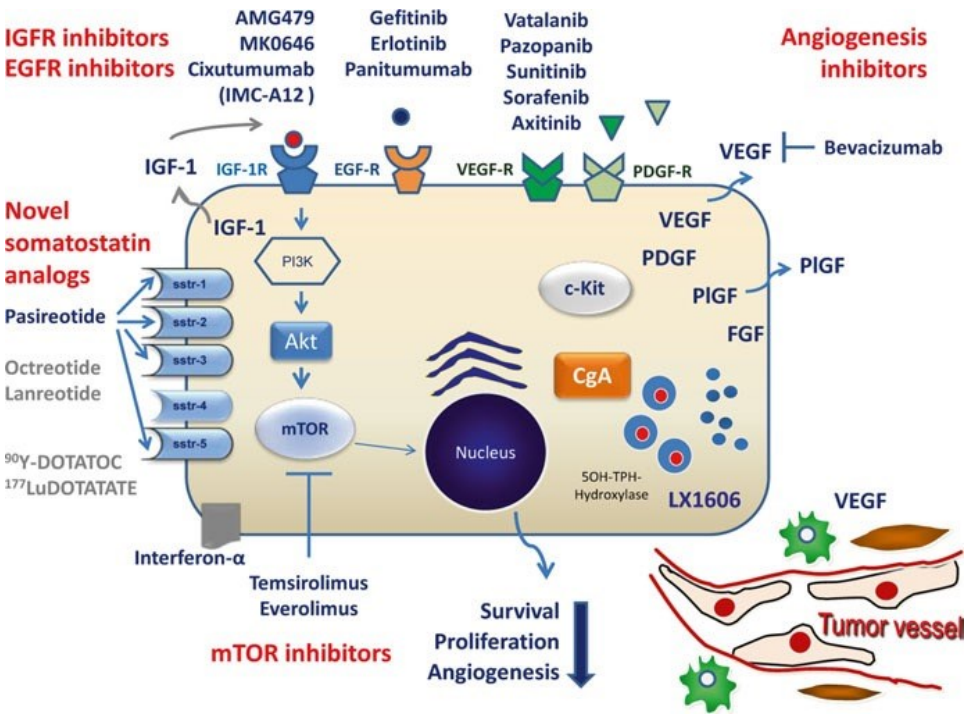

Figure 2: *Signaling pathways, drug targets and targeted therapies in NET. As reported from Pavel et al.([159])*

Anti-VEGFR therapy: NETs are highly vascularised and extensively express proangiogenic molecules such as VEGF and VEGF receptor, hypoxia inducible factor 1a and microvessel density ([160]). The antiangiogenic compounds currently used in clinical practise in advanced NETs include monoclonal antibodies against VEGF, such as bevacizumab and small molecules that inhibit the tyrosine kinase receptors domains (RTK) of the VEGFR, such as

Sunitinib, Sorafenib, Pazopanib and Valatinib. Pazopanib, is an oral RTK inhibitor of VEGFR-1, -2 and -3, PDGF-ß and c-kit that has been used for sequencing treatment in progressive metastatic NET and has showed a clinical benefit (defined as complete response, partial response, and stable disease at 6 months) in 85% of patients. Sorafenib is currently approved for the treatment of hepatocellular carcinoma and renal cell carcinoma, and it has shown modest activity in metastatic GEP-NETs ([145]). Further antiangiogenic drugs are evaluated in combination with SSA or systemic chemotherapy. Despite these advances, some tumors show intrinsic resistance to antiangiogenic therapies, whereas acquired resistance develops in others.

Anti-EGF and anti-IGF therapy: the EGF receptor is frequently expressed in NETs and the binding of EGF or TGF-α induces RAF/MAP/ERK signalling in tumor cells; however, mutations of the EGFR tyrosine kinase which are predictive of a response to EGFR tyrosine kinase inhibitors in other types of cancers are rather uncommon in NET ([161]).
In vitro and in vivo studies using the EGF receptor inhibitor Erlotinib suggested a potential role and currently phase II studies are on going ([162]). Activation of the IGF-1R by IGF-1 and IGF-2 plays an important role in tumor cell proliferation, and in NET cell lines, it has been demonstrated that IGF-1 stimulates tumor cell growth by an autocrine loop. IGF-dependent activation of IGF-1R, highly expressed in GEP-NETs increases growth and liver metastases of GEP-NETs ([163]) and represents an additional drug target. Preclinical studies demonstrated that NVP-AEW541, a selective IGF-1R inhibitor, induces apoptosis and cell cycle arrest in human NET cell lines and primary cultures ([164]). IGF-1 also plays a role in the upstream activation of the mTOR pathway ([165]).

Inhibitors of the mTOR Pathway: mTOR is an intracellular Ser/Thr kinase that plays an important role in multiple signalling pathways. It is involved in cell survival, proliferation and metabolism, integrating response to glucose, growth factors (IGF-1) and hormones ([166]). The main signalling pathway by which mTOR is activated is the PI3K/AKT/mTOR cascade, identified as an attractive target for anticancer activity. mTOR activity is associated with pathogenesis and aggressiveness of P-NETs in which mutations or deregulated expression of upstream regulators of mTOR (PTEN, TSC2) have been reported ([167]). Temsirolimus and Everolimus are inhibitors of mTOR structurally related to rapamycin. Temsirolimus was the first mTOR inhibitor used in NET patients in a phase II study in advanced progressive NETs ([168]), while Everolimus has been recently approved by FDA and EMA, for the treatment of advanced, well-and moderately differentiated P-NETs

based on two multicentre placebo-controlled randomized trials ([169]), improves the management of these tumors demonstrating the great importance of molecular research and translational medicine.

1.6 PROGNOSIS

Although the prognosis of NETs is usually better than carcinomas arising from the same organs, precise estimates are difficult to assess because of a poor understanding of their natural history and the diverse and often empiric choice of treatment. Typically, 5-year survival rates for localised lesions, is of 87-89% for typical bronchial carcinoids, 44-78% for atypical bronchial carcinoids, 65% for ileal primaries, 90% for rectal tumors, and this diminishes markedly with distant metastases disease. For P-NETs, 5-year survival for local disease is 79% dropping to 27% with distant disease ([34]).

1.7 EVEROLIMUS AND PI3K/AKT/mTOR IN NETs

1.7.1 THE PI3K/AKT/mTOR PATHWAY

The target of rapamycin (TOR) was originally discovered in the yeast Saccharomyces cerevisiae, as a target of the macrolide fungicide rapamycin; the structurally and functionally conserved mammalian counterpart (mTOR) was subsequently discovered biochemically based on its rapamycin inhibitory properties ([170]). Due to the strong homology between its C-terminus and the catalytic domain of PI3K, mTOR was included inside the PI3K-related protein kinase family (PIKKs) ([171]). It plays a critical role in several cell-signalling pathways, which promote tumorigenesis through the phosphorylation of proteins that directly regulate protein synthesis, cell-cycle progression, cell growth, cell survival, metabolism, autophagy, and proliferation. mTOR exists s two distinct complexes, mTORC1 and mTORC2; whereas mTORC1 complex is strongly inhibited by rapamycin, mTORC2 is not affected by the drug ([172]). mTORC1 is a heterotrimeric protein kinase that consists of the mTOR catalytic subunit, raptor a regulatory associated protein of mTOR that might have roles in mTOR assembly, recruiting substrates to mTOR, and in regulating mTOR activity, mLST8 (also known as GβL), and two negative regulators, PRAS40 and DEPTOR ([173]). In response to growth

factors and nutrients, it is activated by the PI3K/AKT pathway and inhibited by the TSC1/TSC2 complex through the suppression of Rheb, a small GTP-binding protein that activates mTORC1. The mTORC1 pathway regulates the protein synthesis and the ribosomal biogenesis through the phosphorylation and subsequent activation of S6K1 (ribosomal S6 kinase 1), and the phosphorylation and inactivation of 4EBP1 (eukaryotic translation initiation factor 4E binding protein 1) ([174]). The mTORC2 complex includes mTOR, rictor (rapamycin-insensitive companion of mTOR), mLST8, mSin1 (also known as mitogen-activated-protein-kinase-associated protein 1, important for mTORC2 integrity and mTOR activity toward AKT Ser473 phosphorylation), Protor, Hsp70 and DEPTOR ([175]). This complex is less understood than mTORC1 but recent works have demonstrated that it directly phosphorylates AKT on Ser473, PKC-α, and paxillin (focal adhesion-associated adaptor protein), regulates the activity of the small GTPases Rac and Rho related to cell survival, migration and regulation of the actin cytoskeleton ([176]). The mTORC1 signaling cascade (Figure 3) is activated by phosphorylated AKT, which in turn, for its activation, requests the activation of phosphatidylinositol 3'-kinase (PI3K) by the tyrosine-kinase receptors (RTKs). These receptors interact with the p85 regulatory subunit of PI3K via the Src homology 2 (SH2) domain of p85. p85 dimerizes with p110 catalytic subunit of PI3K and localizes the p85/p110 heterodimer to the plasma membrane. Upon activation, the p110 subunit of PI3K phosphorylates phosphatidylinositol-4-5-bisphospate (PIP2) to the active second messanger (PIP3), which recruits AKT to the plasma membrane resulting in a conformational change and its activation ([177]). To be activated, AKT needs two phosphorylation simultaneously on Thr308 and Ser473 residues, induced by PDK1 and mTORC2 respectively ([178]). Activated mTORC1 phosphorylates downstream effectors, including p70S6K1 and 4EBP1; S6K1 phosphorylates the 40S ribosomal protein S6, enhancing the translation of mRNAs with a 5'-terminal oligopolypyrimidine (5'-TOP); the target of S6K1 include ribosomal proteins, elongation factors and insulin growth factor 2 ([179]). 4EBP1 inhibits the initiation of protein translation by binding and inactivating eIF4E (eukaryotic translation initiation factor 4E) ([180]), but its phosphorylation promotes the dissociation of eIF4E from 4EBP1, relieving the inhibitory effect of 4EBP1 on eIF4E-dependent translation initiation, enabling cap-dependent protein translation, and inducing increased translation of mRNAs with regulatory elements in the 5'-untranslated terminal regions (5'-UTR) of its downstream target genes (e.g. c-myc, ornithine decarboxylase and Cyclin D1), which are required for G1-to-S phase transition ([181]).

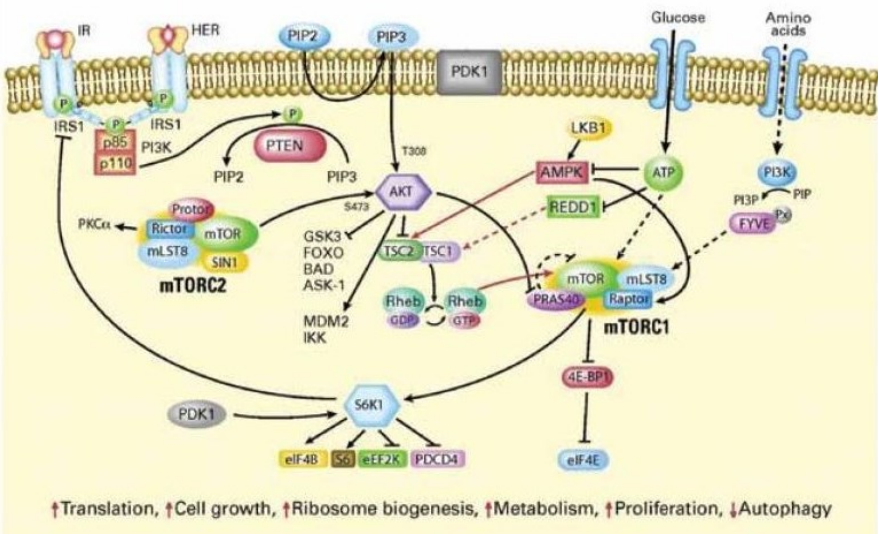

Figure 3: *The PI3K/AKT/mTOR signalling pathway.*

1.7.2 THE mTOR PATHWAY AND CANCER

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 ([182]). In human cancers, several mutations have been identified in mTOR gene resulting in a constitutive activation of mTOR signalling ([183]). In spite of this, other signalling components upstream and downstream of mTORC1 are frequently altered in human tumors (Table 5).

Proto-oncogenes	Alterations described	References
AKT	*AKT* is amplified in a subset of human cancers, such as breast and ovarian cancers.	[126]
4EBP1	4EBP1 expression was found to be associated with poor prognosis in several human tumours, such as breast, colon, ovarian and prostate cancers. The phosphorylation of 4EBP1 was also found to be associated with chemoresistance in ovarian cancer.	[127–129]
eIF4E	Ectopic overexpression of eIF4E can transform cells *ex vivo* and *in vivo*. eIF4E is overexpressed in many human tumours, such as breast, colon, and head and neck cancers, non-Hodgkin's lymphomas, and chronic and acute myelogenous leukemias.	[130,131]
PI3K	High *PI3K* activity was implicated in cell transformation and tumour progression and described in several human cancers, such as ovarian, gastrointestinal, breast and prostate cancers.	[126, 132–134]
Rheb	Rheb overexpression is described in many tumour cells, and Rheb upregulation is critical for squamous carcinoma and associates with poor prognosis in breast and head and neck cancers.	[135,136]
S6K1	S6K1 is overexpressed in in lung and ovary cancers and its expression correlates with poor prognosis in breast, kidney and hepatocellular carcinomas.	[137–142]
Tumour suppressor genes		
LKB1	Individuals with mutations in *LKB1* develop Peutz-Jeghers syndrome, which includes the occurrence of gastrointestinal tract hamartomas.	[111,143]
PTEN	Loss of *PTEN* function has been described in a large proportion of advanced human cancers, such as melanoma, breast, prostate and renal cancers. Individuals with inherited mutations in *PTEN* develop hamartoma tumour syndromes (Cowden disease, Bannayan-Riley-Ruvalcaba syndrome, Proteus syndrome, Lhermitte-Duclos disease) and are at higher risk for developing several cancers.	[124,132, 143–146]
TSC1/TSC2	Patients with mutations in *TSC1* or *TSC2* develop tuberous sclerosis complex (TSC), a syndrome that includes the development of hamartomas in many organs. Mutations in *TSC2* may also lead to the development of Lymphangioleiomyomatosis (LAM).	[147–149]

Table 5: *Proto-oncogenes and tumor suppressor genes linked to the mTOR pathway, as reported from Populo et al. ([179]).*

In cancer cells often receptor tyrosine kinases (RTKs) such as HER-2 (human epidermal growth factor receptor 2) and IGFR (insulin-like growth factor receptor) are overexpressed or aberrantly activated, triggering multiple cytoplasmic kinases including serine/threonine kinases and promoting cancer development ([179]). Phosphates and tensin homologue (PTEN), the negative regulator of PI3K signalling, decreases its expression in a spectrum of cancers, including prostate, breast, lung, melanoma, endometrial, thyroid, brain cancers and renal carcinoma, and several mechanisms, including mutation, loss of heterozygosity, methylation, aberrant expression of regulatory microRNA, and protein instability may determine its downregulation ([184]). Furthermore, AKT and PI3K amplifications have been

found in human cancers ([185]). PI3K amplifications have been found in 40% of ovarian and cervix cancers, in correspondence of the genomic region containing PI3K3CA, which encodes the p110 alpha catalytic subunit of PI3K ([186]). It was also shown that the expression of genes in the mTOR pathway is altered in most P-NETs, in particular loss of function in TSC1 and TSC2 tumor suppressor genes that inhibit mTOR, has been associated with the development of P-NETs ([187]). Moreover it was observed that PTEN is down regulated in approximately 75% of P-NETs and the low expression is associated with shorter disease-free and overall survival ([188]).

1.7.3 mTOR INHIBITORS AND NETs

mTOR inhibitors used in clinical practice are derived from Rapamycin (Sirolimus, Wyeth, Madison, NJ, USA), a macrocyclic lactone isolated from the bacterium Streptomyces hygroscopicus. It was identified as an antifungal agent, and in 1999 was approved by the FDA as an immunosuppressor after organ transplantation ([189]). Preclinical studies indicate that Rapamycin also reduces or arrests the growth of rhabdomyosarcoma, glioblastoma, small cell lung cancer, osteosarcoma, pancreatic cancer, breast cancer, prostate cancer, and B-cell lymphoma cell lines ([190]).

Several derivates of Rapamycin, with more favourable pharmacokinetic and solubility properties have been synthesized; these include Temsirolimus (CCI-779 Wyeth, Madison, NJ, USA), Everolimus (RAD001 Novartis, Novartis, Basel, Switzerland), Deforolimus (AP23573 ARIAD, Cambridge, MA, USA) and 32-deoxorapamycin (SAR943) or Zotarolimus (ABT-578 Abbott Laboratories, Abbott Park, IL, USA) ([179]). Rapamycin and its derivates bind to FK-506-binding protein-12 (FKBP-12) to inhibit the kinase activity of mTOR. This complex interacts with mTORC1 thereby inhibiting the activation of the phosphoprotein kinase, and reducing the activity of the downstream effectors S6 ribosomal protein kinase (S6K1) and eukaryotic elongation factor 4E-binding protein (4EBP1) ([186]) (Figure 4). Subsequently, Everolimus produces the inhibitory effects on tumor cell proliferation and angiogenesis. Of which, the latter is mediated by inhibiting hypoxia-inducible factor 1-alpha (HIF-1α) expression ([191]). The FKBP12-rapamycin complex cannot bind directly to mTORC2, although prolonged treatments can disturb mTORC2 assembly and inhibit the phosphorylation of its downstream substrate AKT on Ser473 ([192]).

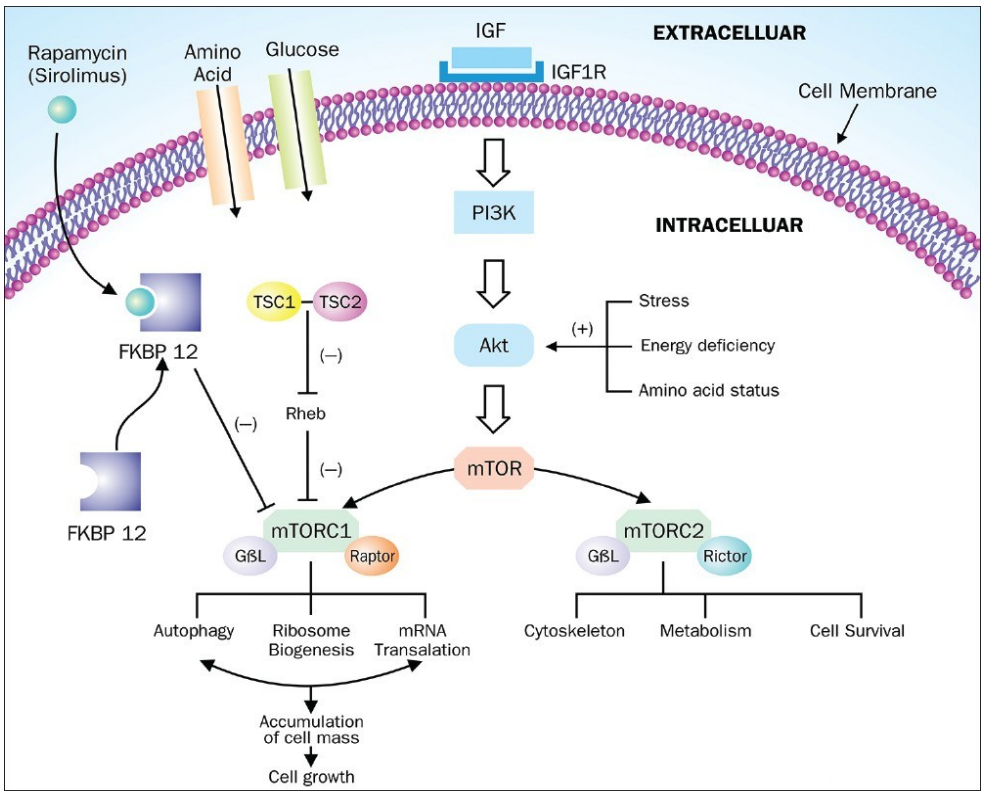

Figure 4: *Mechanism of action of Rapamycin and its derivates, as reported from Madke 2013, Indian Dermatol Online J ([193])*

Rapamycin and its analogues Temsirolimus, Everolimus and Deforolimus are currently being evaluated in clinical trials for cancer treatment ([194]). It was demonstrated that these compounds have cytostatic activity as a single agent in animal models and have synergistic effects in combination with conventional cytotoxic agents, with tamoxifen or with radiation. In clinical studies, these drugs have shown activity in many solid cancers ([195]); encouraging results have also been obtained in a subset of cancers such as Hodgkin lymphoma, non-Hodgkin's lymphoma and breast cancer ([179]). Noteworthy, in phase II clinical studies, Temsirolimus has been shown to have effects in patients with renal cell carcinoma (RCC) and glioblastoma ([196]). *In vitro* and *in vivo* studies had showed that Everolimus reduced cell proliferation, and angiogenesis; in human pancreatic BON1 cells, it exerted a potent dose-dependent inhibition of cell growth involving G0/G1 phase arrest as well as induction of apoptosis ([197]). Likewise, Everolimus has recently shown promising antitumor activity in two phase 2 studies involving patients with pancreatic neuroendocrine tumors. For this reason, FDA approved it for the treatment of advanced well and moderately differentiated P-NETs. Moreover, in a phase III trial RADIANT-3 it was determined whether Everolimus, at the dose of 10 mg per day, would prolong progression-

free survival (PFS), as compared to placebo. It was shown that the median PFS was 11.0 months with Everolimus as compared with 4.6 months with placebo, with a 65% reduction in the estimated risk of progression or death ([198]). Although the molecular pathogenesis of sporadic P-NET is unknown, down-regulation of TSC2 and PTEN is frequent and leads to deregulation of the mTOR pathway. Low TSC2 and PTEN levels are associated with cancer progression, an increased rate of proliferation (as assessed by Ki-67 index) and shortened progression-free and overall survival ([199]). In a study of paired biopsy specimen, it was seen a reduced tumor proliferation in P-NETs after Everolimus treatment, as evidenced by a decreasing percentage of cells with Ki-67 labeling ([200]). Therefore, the clinical benefit observed after Everolimus treatment, confirms the importance of the mTOR pathway in pancreatic neuroendocrine tumors. The antiproliferative effects of Everolimus had also been observed in bronchial carcinoids primary cultures ([201]), in which a significant reduction of cell viability (≈ 30%) was observed in 67.5% of samples, as well as an inhibition of p70S6K and a reduction of CgA and VEGF secretion. Moreover, in a phase 3, randomized, placebo-controlled RADIANT-2 study, an improvement of PFS was observed also in patients with advanced lung NET ([202]). Although most of rapalogs are well tolerated in clinical, some toxic effects can arise, including skin reactions, stomatitis, thrombocytopenia, diarrhoea, fatigue, hyperlipidaemia and hyperglycaemia while, and more rarely renal insufficiency, peripheral edema, interstitial pneumonitis and infections ([203]). To improve the response rates, multiple combination therapies of Everolimus with somatostatin analogues or angiogenesis inhibitors and dual inhibitors targeting upstream and downstream signalling of the mTOR pathway are currently under investigation. Since other treatments are available (somatostatin analogues, chemotherapy with either Temozolomide or Streptozotocin + 5-fluouracil, angiogenesis inhibitors), it is important to define which patients can benefit most of targeted therapy with Everolimus and in which sequence it should be used.

1.7.4 RESISTANCE TO mTOR INHIBITORS

Despite the demonstrated efficacy of Everolimus in prolonging PFS in a significant number of patients with advanced P-NET ([198,204]), and bronchial carcinoids ([202]), some patients do not benefit from Everolimus treatment, probably due to the development of primary or secondary acquired resistance to this drug ([205]). However, the precise mechanism underlying Everolimus resistance remains unknown. As shown in figure 5,

Everolimus inhibits mTOR activity by interacting with the mTORC1 complex but do not affect mTORC2. mTORC2 is a positive regulator of AKT, and selective inhibition of mTORC1 by Everolimus results in an increase in mTORC2 activity and in compensatory increase in AKT phosphorylation at Ser473 ([206]). This rebound AKT activation has been proposed as one of the potential mechanism of resistance to Everolimus. Another postulated mechanism of resistance is via IGF-1/IGF-1R signalling due to inhibition of the S6K negative feedback ([207]). Insulin receptor substrate-1 (IRS-1) is normally phosphorylated by p70S6K and therefore under basal negative regulation; mTOR inhibition prevents IRS-1 phosphorylation thus allowing IRS-1 to complex with IGF-1R and promotes AKT signalling ([208]), thereby generating another positive feedback loop accounting for resistance.

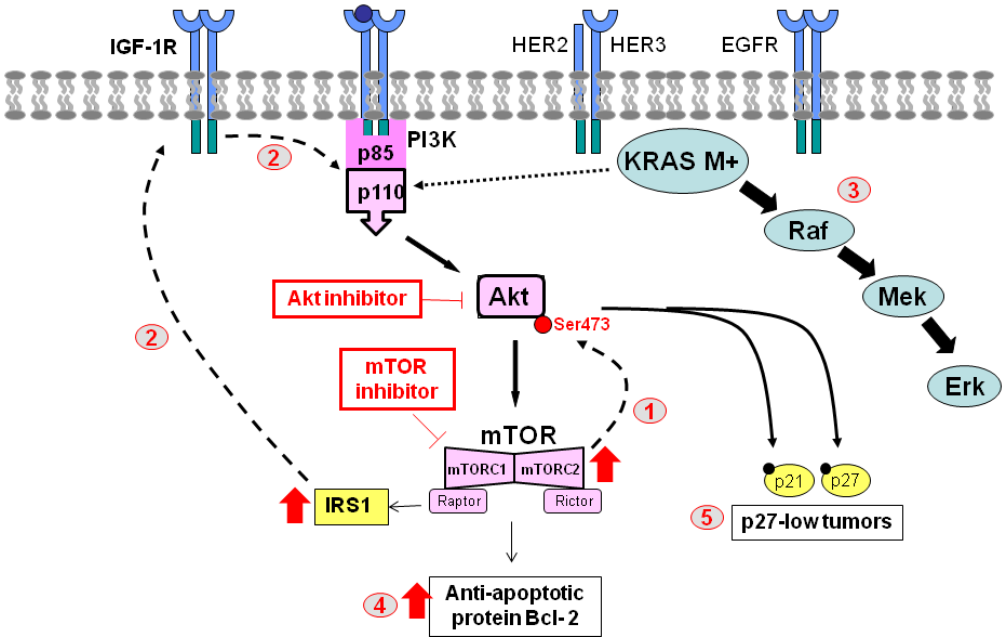

Figure 5: *Proposed mechanisms accounting for resistance to inhibitors of the PI3K/AKT/mTOR pathway, as reported from Cheaib et al. ([209])*

Previous studies have shown that BON1 cell line, displays constitutive activation of the AKT/mTOR pathway due to an autocrine IGF-1 loop ([210]). Similar findings have also been shown in a rat insulinoma cell line, where Everolimus was able to inhibit TSC2, mTOR and p70S6K, but not AKT phosphorylation, with no additive effects when used in combination with SSA analogues ([211]). In an additional *in vitro* study, assessed by Moreno et al. ([212]) on two NET cell lines, BON1 cell line and NCI-H727 cell line, derived from a human bronchial carcinoid, it was found that cell proliferation was significantly reduced by

rapamycin treatment as a single agent, altering the PI3K/AKT signalling pathway by inhibiting S6K1 and 4EBP1 phosphorylation with feedback loop AKT activation. Since an evident association between the expression levels of PI3K/AKT/mTOR components and response to Everolimus in vitro was observed in bronchial carcinoids and in non-functioning pituitary adenomas ([201,213]), it is reasonable to speculate that the analysis of this pathway may be useful to predict the response to mTOR inhibitors. Biopsies in patients with NETs have shown that baseline AKT activation is associated with an aggressive clinical course, but also with an increased PFS under Everolimus and Octreotide treatment ([214,215]). Similarly, it has been previously demonstrated that phosphorylated mTOR protein levels may differentiate human bronchial carcinoids sensitive from those that are resistant to Everolimus treatment in vitro. In addition, basal phosphorylated mTOR, p70S6K, AKT and ERK 1/2 are expressed at higher levels in BCs responder to Everolimus in vitro, as compared to those resistant ([213]). Moreover, higher mTOR expression and activity have been found in foregut than in midgut NETs ([216]). The expression of phospho-mTOR and its downstream targets had been reported to be significantly different between low-intermediate grade tumors (typical and atypical carcinoids) and high-grade tumors (large cell neuroendocrine carcinomas and small cell lung cancers) ([217]), and in poorly differentiated GEP-NETs as compared to well differentiated ([218]). The PI3K/AKT/mTOR pathway may be activated upstream also by a mutated and constitutively activated RAS/MAPK pathway. In addition, somatic mutations have also been investigated to possibly predict sensitivity or resistance to mTOR inhibitors. Previous studies have demonstrated that mutations in the PI3K pathway components were found in human cell lines in which rapalogs determine an antiproliferative effect ([219]). Mutations in PTEN gene, leading to a reduced protein expression, have also been reported to characterize the NET cell lines sensitive to the antiproliferative effects of rapalogs, ([214]), in particular P-NETs especially those showing an aggressive clinical behaviour ([199]). Other genetic mutations have been evaluated as putative biomarkers of sensitivity to rapalogs. In P-NET patients, a Single-Nucleotide Polymorphism (G388R) in the Fibroblast Growth Factor Receptor isoform 4 gene (FGFR4) has been reported to be associated with local invasiveness, lymphovascular invasion, lymph nodal and metastases and with lower response to Everolimus in vivo, but the role of this mutation as a predictive marker of rapalogs sensitivity is still controversial ([220]). Therefore, since NETs display few relevant mutations, DNA profiling may not help in predicting therapeutic responsiveness.

1.8 TGF-β AND NETs

1.8.1 TGF-β FAMILY

The Transforming Growth Factor (TGF-β) superfamily represents a large family of over 35 structurally related pleiotropic cytokines in vertebrates that includes TGF-β1, TGF-β2, TGF-β3, activins, inhibins, and bone morphogenetic proteins (BMPs) ([221]). Members of the TGF-β family exert a wide range of biological effects on a large variety of cell types; they play an important role in regulating several biological processes, such as cell growth, differentiation, matrix production, migration, and apoptosis. Many of them have important functions during embryonal development in pattern formation and tissue specification; in the adult they are involved in tissue repair and modulation of the immune system. Perturbation of their signalling has been implicated in several developmental disorders and in various human diseases including cancer, fibrosis and autoimmune disease ([222]). TGF-β1, TGF-β2, and TGF-β3 are encoded by specific genes and expressed either in tissue-specific manner that regulated during development. TGF-β1 is specific of endothelial cells, hematopoietic and connective tissue, TGF-β2 of neuronal and epithelial cells, while the TGF-β3 is expressed in mesenchymal cells. The three isoforms have highly conserved sequences with a homology of 70-80% ([223]). TGF-β1, which is the prototype of this family, is encoded on Chr19q13.1 and it is a 44.3 kDa protein that is usually secreted into the extracellular matrix as in an inactive form (Figure 6). The TGF-β1 precursor contains 390 amino acids with an N-terminal signal peptide of 29 amino acids required for secretion from a cell, a 249 amino acid pro-region (latency associated peptide or LAP), and a 112 amino acid C-terminal region that becomes active TGF-β1 upon activation by proteolytic cleavage ([224]). Both LAP and TGF-β1 exist as homodimers in circulation, but the disulphide-linked homodimers of LAP and TGF-β1 remain non-covalently associated, forming the small latent TGF-β1 complex (SLC, 100 kDa). The large latent TGF-β1 complex (LLC, 235–260 kDa) contains a third component, the latent TGF-β binding protein (LTBP), which is linked to LAP by a single disulphide bond. The LTBP does not confer latency but allows for efficient secretion of the complex to extracellular sites. The release of the active form, which is necessary for its biological activity, requires proteolytic cleavages involving matrix metalloproteinases, alterations in pH, production of reactive oxygen species or the activity of thrombospondin-1 ([225]).

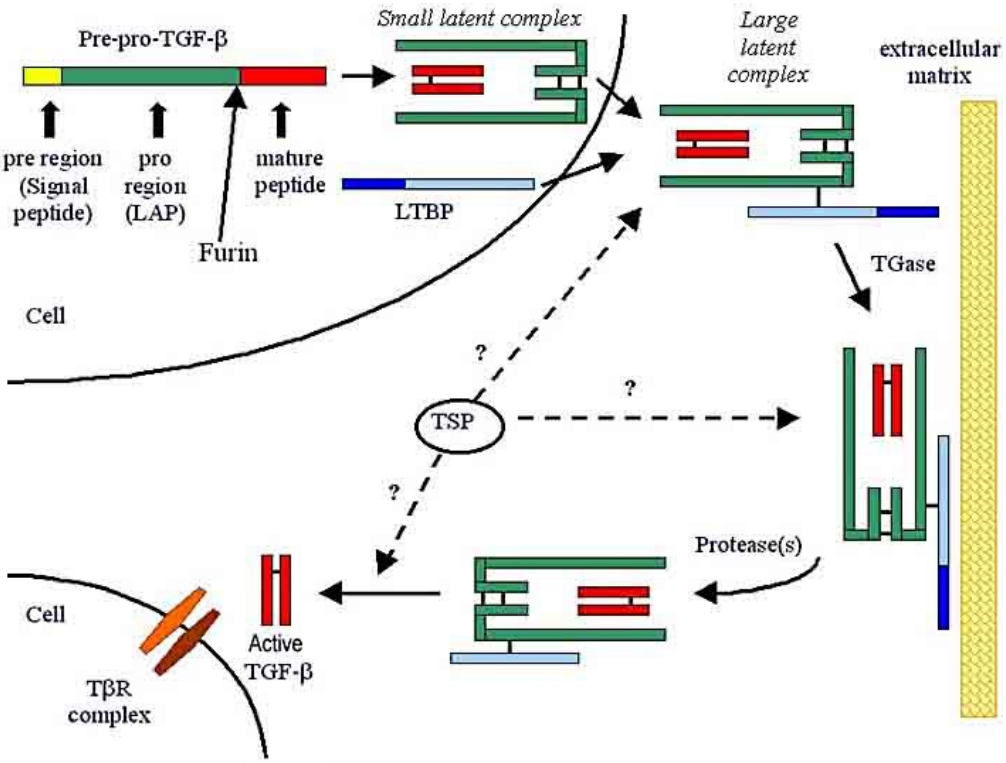

Figure 6: *Mechanism of processing of latent transforming growth factor-beta, as reported from Gressner et al. ([226]). TGase: Tissue transglutaminase; TSP: Thrombospondin*

In its active form, TGF-β1 is a 25 kDa protein composed of two polypeptide chains of 112 amino acids residues, each with seven highly conserved Cys residues, six of which form a structure known as "cysteine" node, while the seventh forms a disulphide bridge with the corresponding cysteine residue of other chain polypeptide ([227]).

1.8.2 TGF-β SIGNALLING PATHWAY

TGF-β family members initiate their cellular action by binding to receptors with serine/threonine kinase activity. This receptor family consists of two subfamilies, type I and type II receptors, which are membrane glycoproteins of 55 kDa and 75 kDa, respectively, structurally similar, characterised by small cysteine-rich extracellular region, a transmembrane domain and a cytoplasmic region consisting mainly of the serine-threonine kinase domain ([228]). Type I receptors, but not type II receptors, have a region of 30 amino acids rich in glycine and serine residues (GS domain) in the juxtamembrane domain, which is involved in the control of kinase activity of the receptor and in its

interaction with the substrate. It was demonstrated that mutations of some serine residues in this domain, resulted in the loss of the ability to transduce the signal ([229]).

TGF-β first binds to the type II receptor (TβR-II), which occurs in the cell membrane in an oligomeric form with activated kinase. Then, the TGF-β type I receptor (TβR-I), which may also occur in an oligomeric form and cannot bind TGF-β in the absence of TβR-II, is recruited into the complex; TβR-II phosphorylates TβR-I in the GS domain to activate it. Ligand binding triggers the assembly of the receptor complex, but it is also stabilized by direct interaction between the cytoplasmic parts of the receptors. This results in the phosphorylation of the Smad family of transcription factors and activation/inhibition of various genes, depending on the state of cell transformation. ([230]). The human genome encodes eight Smad family members; they are ubiquitously expressed throughout development and in all adult tissues ([231]), and many of them (Smad 2, Smad 4, Smad 5, Smad 6, Smad 8) are produced from alternatively spliced mRNAs (Gene encyclopedia, GeneCards). Functionally, Smads can be subdivided into three subfamilies: receptor-activated Smads (R-Smads), Smad 1, Smad 2, Smad 3, Smad 5, Smad 8, which are directly phosphorylated by TβR-I; common mediator Smads (Co-Smads), Smad 4, which oligomerise with activated R-Smads, and inhibitory Smads (I-Smads), Smad 6 and Smad 7, which exert a negative feedback effect by competing with R-Smads for receptor interaction and by marking the receptors for degradation ([221]). Smads are molecules of 42-60 kDa with two regions of homology at the amino and carboxy terminals, termed Mad-homology domains, MH1 and MH2, respectively, which are connected with a proline-rich linker sequence (figure 7). MH1 domain regulates nuclear import and transcription by binding to DNA and interacting with nuclear proteins, the MH2 domain regulates Smad oligomerisation, recognition by TβR-I and interacts with cytoplasmic adaptors and several transcription factors ([232]).

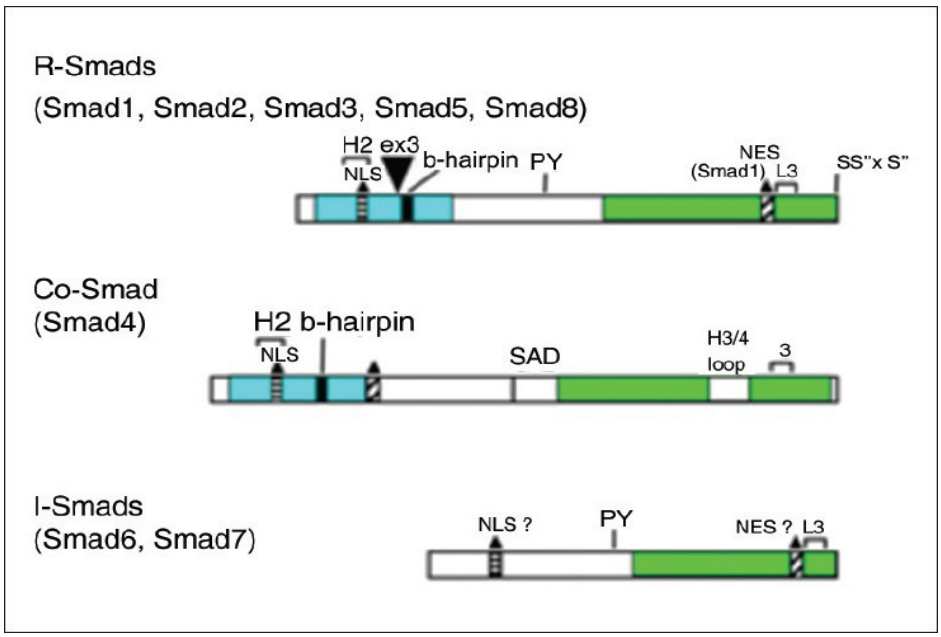

Figure 7: *Diagrammatic representation of the three subfamilies of Smads as reported from Moustakas et al. ([232]). The MH1 domain is coloured in blue and the MH2 domain in green. Domains and sequence motifs are indicated as follows: α-helix H2, L3 and H3/4 loops, β-hairpin, the unique exon 3 of Smad 2 (ex3), NLS and NES motifs, the proline-tyrosine (PY) motif, of the linker recognised by Smurfs, the unique SAD domain of Smad 4 and the the SSXS motif of R-Smads with asterisks indicating the phosphorylated serine residues.*

Access of the R-Smads to TβR-I is facilitated by auxiliary proteins such as Smad anchor for receptor activation (SARA), which restrains them into the cytoplasm in the basal state ([233]). Phosphorylation of the C-terminal serine residues in R-Smads by TβR-I is a crucial step in TGF-β family signalling (Figure 8). TGF-β phosphorylates Smad 2 and Smad 3, with as consequence, the formation of oligomeric complexes with the Co-Smad, Smad 4. These complexes translocate and accumulate in the nucleus, where they control gene expression in a cell-type-specific and ligand dose-dependent manner through interactions with transcription factors, coactivators and corepressors ([222]). Previous studies have demonstrated that nuclear translocation of R-Smads is independent of Smad 4, whereas translocation of Smad 4 after TGF-β signalling requires the presence of an activated R-Smad ([234]). The nuclear import mechanisms of R-Smads have been analysed in detail; the MH1 domains of all eight Smads each contains a lysine-rich motif that has been shown to act as a nuclear localisation signal (NLS). In addition, in Smad 3, C-terminal phosphorylation induces a conformational change that exposes the NLS to importin-β that mediates the nuclear import ([235]). Smad 6 and Smad 7 act in an opposing manner to R-

Smads and antagonize signalling. They were originally shown to compete with R-Smads for binding to activated TβR-I and thus to inhibit the phosphorylation of R-Smads. Subsequently, they were found to recruit E3-ubiquitin ligases, known as Smad ubiquitination regulatory factors, Smurf1 and Smurf2, to the activated TβR-I, resulting in receptor ubiquitination and degradation, and termination of signalling ([233]). Recently, Smad 7 has been shown to recruit a complex of GADD34 and the catalytic subunit of protein phosphatase 1 to the activated TβR-I to dephosphorylate and inactivate it ([236]). Furthermore, not only are levels of Smads in the nucleus important, but also their duration of residence is significant. Epithelial cells with a sustained TGF-β-Smad response are sensitive to TGF-β-induced growth arrest, whereas pancreatic tumour cells demonstrating a transient Smad response specifically evade TGF-β-induced growth arrest, although they maintain other TGF-β responses ([237]).

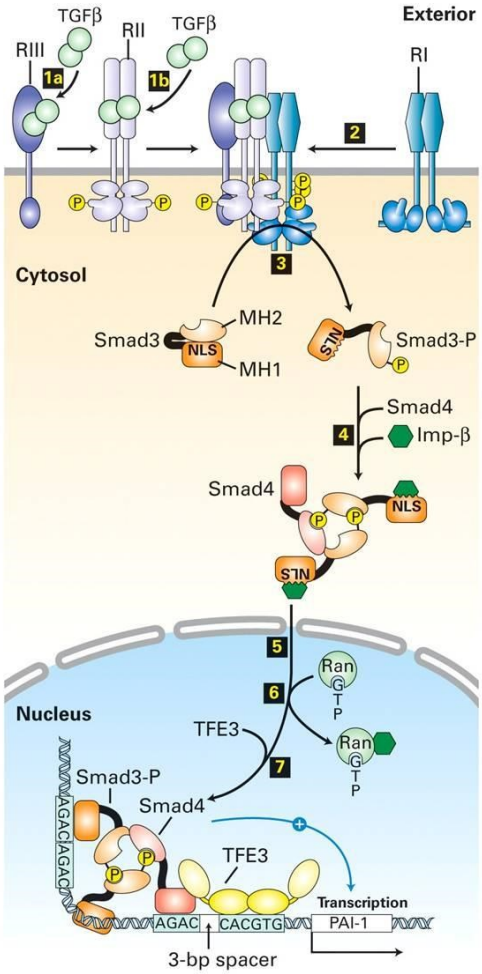

Figure 8: *The basic Smad pathway*

TGF-β utilizes a multitude of intracellular signalling pathways in addition to Smads to regulate several cellular functions. These non-Smad pathways include various branches of MAP kinase (MAPK) pathways, Rho-like GTPase signalling pathways and PI3K/AKT pathways ([238]) (Figure 9). Non-Smad signalling pathways directly modify (e.g. phosphorylate) the Smads and thus modulate the activity of the central effectors; Smads directly interact and modulate the activity of other signalling proteins (e.g. kinases), thus transmitting signals to other pathways; and the TGF-β receptors directly interact with or phosphorylate non-Smad proteins (e.g PI3K), thus initiating parallel signalling that cooperates with the Smad pathway in eliciting physiological responses ([239]).

In particular, it was shown that TGF-β induces the stress-activated kinases p38 and JNK (Jun N-terminal Kinase), which have a synergistic effect to Smad signalling in leading to apoptosis and epithelial-mesenchymal transition (EMT). TGF-β can also signal through the mitogen activated protein kinase (MAPK) pathway by activating the extracellular-signal-regulated kinases 1 and 2 (ERK1 and ERK2), further inducting EMT. Rho GTPases have been shown to relay the TGFβ signals leading to cytoskeleton reorganization, cell motility, and invasion, through activation of RhoA, Cdc42, and Rac. Finally, TGF-β is also able to signal through thePI3K/AKT pathway to inhibit cell growth and induce EMT ([240]).

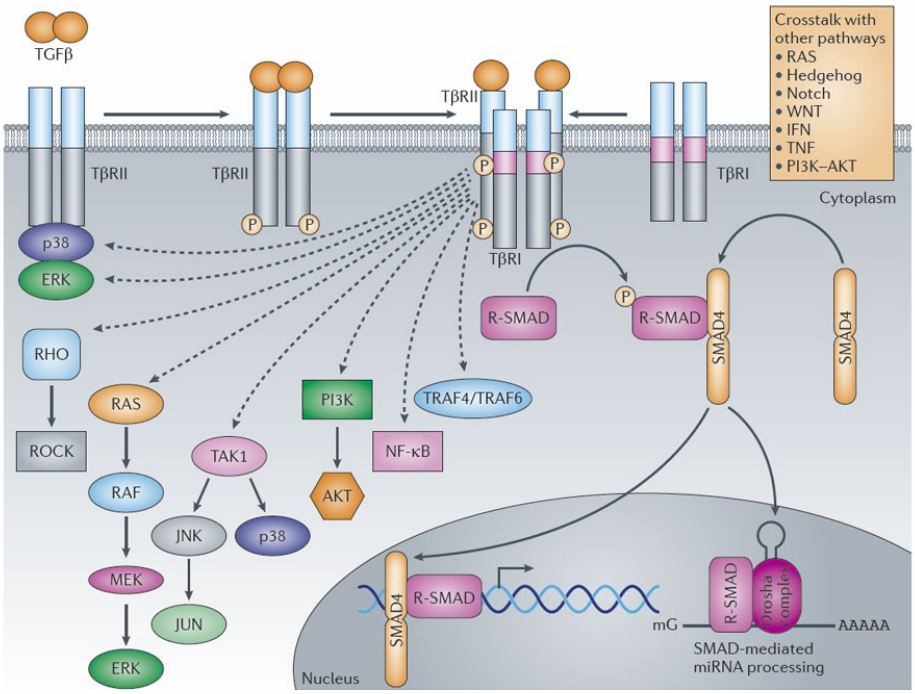

Figure 9: *Schematic representation of non-canonical TGF-β signalling and crosstalk with other signalling pathways, as reported from Akhurst et al. ([241])*

1.8.3 TGF-β AND CANCER

TGF-β and its receptors are widely expressed in all tissues and its biological and physiological functions, ranging from cell growth, differentiation, apoptosis, migration, adhesion, embryogenesis, hormonal synthesis and secretion, bone formation, erythropoiesis, reproduction, immunity, tissue remodelling and repair, are of crucial importance in human diseases, particularly cancer ([242]).

TGF-β plays a dual and paradoxical effect on cell growth and metastases: accelerating proliferation in some cell types, such as fibroblast cells, while suppressing proliferation in ephitelial-, immune-, and neural-derived cells ([243]). Additionally, it was shown that in the early stages of epithelial or neural cells derived tumor development, TGF-β works as a suppressor of tumor proliferation and metastasis, and later works as an accelerator of these processes, which include induction of Epithelial-Mesenchymal Transition (EMT), cell adhesion, migration, invasion, chemo-attraction, and tumor metastasis ([244]). TGF-β exerts its suppressive effects by inhibiting cell cycle in the G1 phase, inducing apoptosis and preventing cell immortalization in numerous target tissues, with a strong cytostatic effect ([245]). This physiological growth restraint by TGF-β is often lost during malignant transformation, which may contribute to the malignant phenotype of various cancers. The loss of physiological responsiveness to TGF-β on carcinogenesis is associated with an elevated expression of TGF-β, somatic mutations of the TβR-I and TβR-II, and loss of function mutations of the Smad genes ([246]). All inactivating mutations or loss of expression of the TGF-β signalling pathway components can be the cause of resistance to TGF-β growth inhibition. Thus, the tumor suppressive effects of TGFβ are observed in normal cells and early carcinomas; conversely, its tumor promoting effects, are more specifically observed in aggressive and invasive tumors. In addition, during tumor progression, they generally produce and secrete a large amount of autocrine TGF-β that is then released in the tumor vicinity; these increased TGFβ levels affect the tumor cells as well as the surrounding stroma by inhibiting cell adhesion, inducing immunosuppression and angiogenesis, and by promoting the degradation of the extracellular matrix, further contributing to the metastatic process ([247]).

The role of TGF-β in neuroendocrine tumor biology is currently unknown. Chaudhry and others have demonstrated TGF-β isoform expression in approximately 50% of mesenchymal and/or tumor cells of GEP-NETs ([248]). Subsequently, it was shown that neuroendocrine tumor cells of the gastroenteropancreatic tract are subject to paracrine and autocrine growth inhibition by TGF-β, which may depend on the low proliferative index of

this tumor entity ([249]). It has been well documented that TGF-β switches from an inhibitor of tumor cell growth to a stimulator of growth and invasion during the late stages of cancer in a variety of tumors; for example while TGF-β inhibits growth in non-transformed colonic epithelial cells, it stimulates proliferation in approximately 50% of colon cancer cell lines ([250]). In another study, comparing normal small intestinal enterochromaffin cells with metastatic human ileal carcinoid cells (KRJ-I cells), it was found that the growth of normal cells could be inhibited by TGF-β, while KRJ-I cells were induced to proliferate by TGF-β ([251]).

1.9 TSC22D1

TGF-β-stimulated clone 22 Domain family member 1 (TSC22D1) also named TSC-22 is a gene localised on chromosome 13q14.11, which is highly conserved during evolution at both the mRNA and protein levels, and found in a range of organisms, from nematodes to flies, birds, fish, amphibians and mammals. The mouse and the rat genes are 100% identical at the amino acid level, while human is 98.5% identical ([252]). It belongs to a superfamily of genes encoding leucine zipper proteins, including TSC22D1, TSC22D2, TSC22D3 and TSC22D4. TSC22D1 was first identified as a TGF-β-inducible gene in mouse osteoblastic cells ([253]) and its expression is induced in a variety of cell lines by TGF-β, epidermal growth factor (EGF), fibroblast growth factor 2 (FGF-2), dexamethasone, tumor necrosis factor α, interferon-γ, interleukin-1β, choleratoxin, lipopolysaccharide, progesterone, serum, phorbol ester, follicle-stimulating hormone and progestin, and moreover, it was observed that TGF-β regulates TSC22D1 post-transcriptionally, through an increase in mRNA stability ([254]). TSC22D1 gene (approximately 145.18 kb), is composed of 18 distinct introns and 7 exons, and was shown to encode a putative transcriptional regulator. It contains a leucine-zipper motif, but it does not have a classic DNA-binding motif at the N-terminal region; it can homodimerize or heterodimerize with other leucine zipper containing transcription factors to activate or repress transcription ([255]). It was hypothesised that TSC22D1 may act as a transcriptional repressor activity, by binding members of the AP-1 family, and inhibiting their DNA binding; and it was found that TSC22D1 heterodimerizes with TSC-22 homologous gene-1 (THG-1) and both act as a transcriptional repressors when fused to DNA-binding domain of transcription factor GAL-4 ([255]). Not much is known about the function of TSC22D1 and its homologues. It has a wide tissue distribution, both in embryonic development and in adults, including the heart, lung, kidney, stomach, intestine, prostate, ovary, brain, and endothelium of blood vessels ([252]). Previous studies have determined its important role in Drosophila embryonic development, in mouse embryogenesis, during which TSC22D1 is up-regulated at sites of ephitelial-mesenchimal interactions and expressed in many neural crest-derived cells ([256]), therefore, it has been proven its involvement in several processes such as epithelial morphogenesis, differentiation, cell growth and in the regulation of cell death ([257]). In addition, TSC22D1 gene regulates the transcription of multiple genes including C-type natriuretic peptide and a single nucleotide polymorphism in the promoter of this gene has been associated with diabetic nephropathy ([258]). Alternatively spliced transcript variants encoding multiple isoforms have been observed for this gene (provided

by RefSeq, Aug 2011): isoform 1 represents the longest transcript and encodes the longest isoform (TSC22D1.1); isoform 2 differs in the 5'UTR, lacks a large portion of the 5' coding region and initiates translation at an alternate start codon; the encoded protein TSC22D1.2 is significantly shorter and has a distinct N-terminus, compared to isoform 1, with a molecular weight of 48 kDa (Figure 10). Isoform 3 lacks a large portion of the 5' coding region and initiates translation at a downstream, in-frame start codon compared to variant 1. Isoform 4 uses an alternate splice site and lacks an exon in the coding region, which results in a frameshift; the encoded protein is shorter and has a distinct C-terminus, compared to variant 1. Variants 3 and 4 encode the same isoform, which has a significantly shorter N-terminus compared to isoform 1 ([259]). Opposite functions of isoform 1 and isoform 2 on cell cycle and survival during mammary gland involution have been demonstrated in vitro; whereas isoform 2 induces cell death and can be up-regulated by TGF-β3 treatment, isoform 1 reduces cell death after TGF-β3 treatment, and increases proliferation. Therefore TSC22D1 isoform 2 may act downstream of TGF-β3 signalling to induce cell death ([257]).

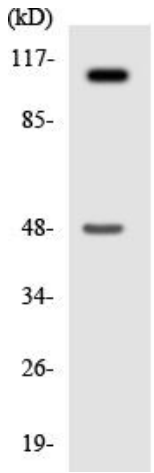

Figure 10: *Molecular weight of TSC22D1.2 isoform*

Given that TSC-22 is a transcription repressor ([255]), it was proposed as putative tumor suppressor gene, because through experiments using TSC-22 siRNA, it was demonstrated that the DNA damage-inducible gene 45 β (Gadd45β) and the tumor suppressor 2 (Lzts2) were putative targets of TSC-22 ([260]). In addition, TSC22D1 participates in growth inhibition of several cancers and its expression was found to be down-regulated or silenced in prostate cancer, breast cancer, colon and gastric cancer, human brain tumor, hepatocarcinoma, cervical cancer and large granular lymphocyte leukemia ([261]), but however the mechanisms underlining its down regulation are largely unknown. In normal

prostate (NP), TSC22D1 protein expression has been found restricted to the basal cell layer of the acinar epithelium, and for this reason, considered a novel basal cell marker. In contrast to NP, prostate cancer (PC) does not show any detectable TSC22D1 protein expression ([262]). This data suggests that the loss of TSC22D1 could be a hallmark of malignant transformation in the acinar epithelium. Furthermore, it has been recently shown a down regulation of its expression also in salivary gland cancer, in which it was observed that the protein is localised in the cytoplasm and that the nuclear translocation of the TSC22D1 protein is a key step in the induction of apoptosis; and in addition it was observed that overexpression of this protein enhances the chemo and radiation sensitivity of salivary gland cancer cells in vitro and in vivo by induction of apoptosis ([263]). Furthermore, mutations in TSC22D1 gene have been associated with the onset of pulmonary adenomas ([264]), and recent published data demonstrate that the overexpression of TSC22D1 was sufficient to reduce cell proliferation, promote cellular apoptosis and inhibit the HDM2-and E6-mediated p53 poly-ubiquitination and degradation in cervical cancer cells; as consequence, the function of p53 was activated ([265]). With regard to TGF-β signalling, it was shown that TSC22D1 enhanced TGF-β signalling pathway by interaction with Smad 4 in human histiocytic leukemia cell line, but how this interaction enhances Smad activity also remains to be determined, probably by recruiting various transcriptional regulators including p300 and HDAC ([266]). Furthermore, TSC22D1 increased levels of Cyclin-dependent Kinase Inhibitor 1A (CDKN1A, also known as p21), a downstream component of TGF-β signalling, in colon carcinoma cells ([267]). Moreover, it was found that TSC22D1 regulated TGF-β signalling through a positive-feedback mechanism; it associates with TβR-I and Smad 7 in mutually exclusive ways and decreases the association of Smad7/Smurfs with activated TβR-I, thereby stabilizing the receptor, preventing ubiquitination, and promoting TGF-β signalling. TSC-22 enhances also TGF-β-induced Smad2/3 phosphorylation and promotes the transcriptional activity of TGF-β ([268]). In addition, in a previous study, the inhibitory effect of TGF-β on cell proliferation, which can be activated by tamoxifen, was reported; it was also found that high levels of TSC22D1 were associated with tamoxifene resistance and with a shorter progression free survival (PFS) and therefore to a worse clinical outcome in breast cancer patients ([269]). The tumor-promoting capabilities of TSC22D1 may be explained by up-regulation of p21 gene. Besides it is a well-known tumor suppressor gene, it was reported that high levels of p21 determined an unfavourable effect in anticancer treatment, probably inducing anti-apoptotic genes ([270]).

2. AIM OF THE STUDY

Neuroendocrine tumors are a spectrum of malignancies arising from neuroendocrine cells spread throughout the body. NETs are very heterogeneous and differ broadly based on organ origin, biological pathway, grade of differentiation, and proliferation rate ([271]). To date, the surgical approach represents the mainstay therapeutic option, and chemotherapy is tried in case of unresectable tumor, even if with controversial efficacy ([272]). In addition, in vitro and in vivo studies have demonstrated that target therapies directed against growth factor receptors and mammalian target of rapamycin (mTOR) could be useful in reducing cell viability and in improving PFS ([213,201,273,198]). Everolimus has demonstrated its efficacy in different neuroendocrine tumors, in particular it has recently shown antitumor activity in advanced, well- and moderately-differentiated P-NETs ([169]), and we have previously demonstrated that approximately 70% of human BC primary cultures respond to Everolimus treatment in terms of cell viability reduction and apoptosis activation ([213]). However, it was shown that a number of patients do not benefit from Everolimus treatment likely due to the development of primary or acquired resistance to this drug ([205]). In addition, in our laboratory, pulmonary NETs microarray data analysis obtained by comparing a pool of low-grade typical carcinoids (TC) tissue specimens with a pool of the more malignant atypical carcinoid (AC) tissues specimens showed a down-regulation of a gene called TGF-β stimulates clone 22 domain family member 1 (TSC22D1) in the ACs samples. This finding was confirmed by real-time PCR and Western blot analysis in our in vitro models of TC (NCI-H727 cells) and AC (NCI-H720 cells).

As the molecular mechanism defining NET behaviour is currently unclear ([274]), a univocal classification is still lacking, although in the last years the researchers were aimed to create clinical-pathological classification to have an appropriate prognostic tool. For these reasons, the aim of our study was to investigate the molecular biology of NETs in order to:

1. Identify new putative predictive markers of sensitivity to Everolimus in human P-NETs primary cultures and tissues in order to overcome resistance to target therapies, selecting patients who may benefit from treatment avoiding useless side effects in resistant patients;
2. Understand the possible role of TSC22D1 as a diagnostic/prognostic tool in BCs by using NCI-H727 cells and human BC tissues in order to develop new therapeutic strategies and improve not only diagnosis but also patient management.

3. MATERIALS AND METHODS

3.1 HUMAN TISSUES COLLECTION

18 primary P-NETs and 2 lymph node metastases, derived from 16 patients (10 males and 6 females; mean age 58.6 ± 4.2 years) operated on at the University of Ancona (Pancreatic Surgery Unit, Department of Surgery) and at the University of Ferrara (Section of Endocrinology and Clinical Surgery) whose tumor characteristics are shown in Table 6, were used for this study. Concerning medical treatments, 13 patients did not receive medical therapy before surgery; 1 patient had been treated with somatostatin analogs (SSA) alone, 1 patient had been treated with SSA in association with chemotherapy, and 1 patient received SSA on association with Everolimus, since after being treated with capecitabine, he showed disease progression. Most P-NETs were diagnosed as G1 or G2 (14/16), displaying a Ki-67 ≤ 10%, with pauci cellular focal necrosis in some cases, without lymph node (11/16) or distant metastases (13/16).

Patient no.	Sex	Age	Hormonal Secretion	Diagnosis	TNM	Stage	Mitosis no.	Ki-67 (%)
1	F	67	NF	NET G2	T3 N1 M1	IV	2/50	8
2	M	74	NF	NEC G3§	T2 N0 M1	IV	42/10	65
3	M	53	NF	NET G2	T2 N1 M0	IIB	2/10	9
4	M	76	NF	NET G1	T1 N0 M0	I	2/10	2
5#	F	38	NF	NET G2	T4 N0 M0	III	2/10	10
6[E]	M	56	NF	NEC G3	T3 N1 M0	IIB	5/10	25
7#[E]	F	65	NF	NET G2	T3 N1 M1	IV	13/10	20
8	F	32	NF	NET G1	T2 N0 M0	IB	1/50	1
9	M	77	NF	NET G1	T1 N0 M0	I	2/10	2
10	F	74	NF	NET G2	T2 N0 MX	IB	2/10	5
11	M	23	Insulinoma	NET G2	T3 N1 M0	IIB	2/10	7
12*	M	65	NF	NET G1	T1 N0 M0	I	2/10	1
13	M	75	Insulinoma	NET G1	T1 N0 M0	I	1/10	2
14	F	65	NF	NET G1	T1 N0 M0	I	2/10	2
15*	M	45	Insulinoma	NET G1	T1 N0 M0	I	0	<2
16	M	53	NF	NET G2	T2 N0 M0	I	n.a.	3

Table 6: *Clinical characteristics of P-NET patients***.** *NET= Neuroendocrine Tumor; NEC= Neuroendocrine carcinoma; NF= Non-functioning; §Poorly differentiated neuroendocrine carcinoma n.a. = not available. # NET patients for whom 1 sample from the primary tissue and 1*

*sample from the lymphnode metastasis were available, each generating a primary culture; [E]NET patients treated with Everolimus in vivo; *NET patients for whom 2 primary tissue samples were available, each generating a primary culture.*

Furthermore, 20 samples, derived from 20 patients (12 males and 8 females, mean age 49.9 ± 18.4 years), whose characteristics are given in Table 7, who were diagnosed with BCs and operated on at the University of Ferrara (Section of Endocrinology, Institute of Surgery) and at the University of Padova (Department of Medical and Surgical Sciences), were included in this study. All patients but two had histological and immunohistochemical diagnosis of typical BC, according to the WHO classification ([67]). Most BCs displayed a Ki-67 <10% (16/20), without regional lymph node involvement (16/20) or distant metastases (20/20).

Patient no.	Sex	Age	Side	Histology	Ki-67 (%)	TNM	PFS (months)
1	M	46	DX	Typical Carcinoid	<1%	T2aN0Mx	36
2	M	38	DX	Not specified	2%	T1aN0Mx	45
3	F	35	DX	Typical Carcinoid	8%	T2aN0Mx	46
4	M	57	DX	Typical Carcinoid	60%	T1bN2Mx	48
5	F	47	SX	Typical Carcinoid	<1%	T1N0Mx	68
6	M	69	DX	Typical Carcinoid	<1%	T2aN0Mx	55
7	M	43	DX	Typical Carcinoid	7%	T2aN2Mx	2
8	F	75	DX	Typical Carcinoid	< 1-2%	pT1aN0Mx	36
9	M	18	DX	Typical Carcinoid	10%	pT1aN0Mx	23
10	M	76	SX	Typical Carcinoid	40%	pT1aN0Mx	34
11	F	61	DX	Typical Carcinoid	4%	pT1aN0Mx	32
12	M	24	DX	Typical Carcinoid	2%	pT1bN0Mx	4
13	M	69	DX	Typical Carcinoid	5%	pT1aN0Mx	/
14	M	34	DX	Typical Carcinoid	1%	T1N0Mx	12
15	M	70	DX	Atypical Carcinoid	10%	T1N2Mx	6
16	M	38	DX	Typical Carcinoid	5%	pT1aN0Mx	33
17	F	77	SX	Typical Carcinoid	1%	pT1aN0Mx	9
18	F	67	SX	Typical Carcinoid	1%	T1aN0Mx	4
19	F	38	DX	Typical Carcinoid	<1%	T2bN1Mx	12
20	F	48	DX	Typical Carcinoid	5%	pT1bN0Mx	10

Table 7: *Clinical characteristics of BC patients.*

For each patient, a tissue fragment was immediately frozen in liquid nitrogen under ribonuclease-free condition and stored at -80 °C until total protein isolation was performed. The remaining tissue sample was collected in culture medium for primary culture studies.

Tissue samples were collected following the guidelines of the local committee on human research. Informed consent of the patients was obtained for disclosing clinical investigation and performing the in vitro study.

3.2 PRIMARY CULTURES

Upon arrival in the Lab, a portion of the fresh tissue was immediately minced in serum-free RMPI-1640 medium (Euroclone Ltd., Wettherby, UK) 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 to separate the dispersed cells and tissue fragments from the larger pieces, and then centrifuged at 800 g, for 5 min at 4°C. After removing the supernatant, tumor cells were resuspended in RPMI 1640 with 10% fetal serum bovine (FBS) and antibiotics (Euroclone Ltd., Wettherby, UK), seeded in 96-well black plates ($1x10^4$ cells/well) and incubated at 37 °C in a humidified atmosphere of 5% CO_2-95% air, as previously described ([201]). After approximately 18 hours, cells were treated with test substances, with further evaluation of cell viability and/or caspase 3/7activity.

3.3 CELL LINE CULTURE

The NCI-H727 cell line, derived from a typical bronchial carcinoid of a 65 years old Caucasian female, was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were grown in RPMI-1640 medium (Euroclone, Milano, Italy), supplemented with 10% fetal bovine serum, at 37°C in a humidified atmosphere with 5% CO_2.

3.4 STABLE TRANSFECTION

Cells were transfected with TSC22D1 shRNA plasmid (OriGene, Rockville, MD, USA) by using TransIT X2® Dynamic Delivery System (Mirus Bio LLC, Madison, USA), an advanced non-liposomal system that comprises of a completely novel class of polymers in addition to other proprietary components that aid in nucleic acid complexation, uptake and endosomal release. This polymeric system efficiently delivers both DNA and RNA out of the endosome and into the cytoplasm overcoming a critical barrier to nucleic acid delivery. Human 4 unique 29mer shRNA constructs in retroviral GFP vector (Gene ID = 8848) (Figure 11) were used. These shRNA constructs were designed against multiple splice variants at the same gene locus.

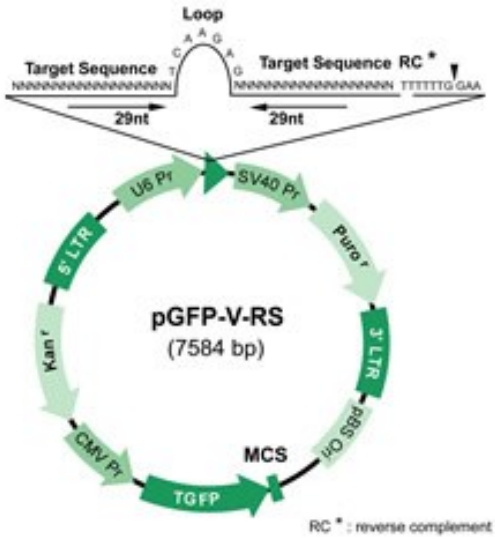

Figure 11: *Map of shRNA Cloning Vector pGFP-V-RS*

Briefly, approximately 24 hours before transfection, the cells were seeded in 6 well plates ($2.5x10^5$ cells/ml) in complete RPMI 1640 medium; cells should be ≥80% confluent at the time of transfection. After overnight incubation, a TransIT-X2:DNA mixture containing 200 µl of Opti-MEM I Reduced-Serum Medium (Thermo Fisher Scientific Inc,Rockford, IL, USA), 2 µg plasmid DNA and 6 µl TransIT-X2 was prepared. The TransIT-X2: DNA complexes were incubated at room temperature for 25 minutes to allow sufficient time for complexes to form, and added drop-wise to different areas of the wells. Then, stably transfected clones were selected by incubation in medium containing puromycin 2 µg/ml

(Sigma-Aldrich, Saint Louis, Missouri, USA). The parental cells are referred as "control", TSC22D1 shRNA transfected cells are referred to as "sh-NCI-H727 cells".

3.5 COMPOUNDS

Everolimus was provided by Novartis; IGF-1 was purchased from PeproTek Inc (Rocky Hill, NJ, 113 USA) and human recombinant TGF-β from R&D Systems (Minneapolis, MN, USA). All other reagents were purchased from Sigma, if not otherwise indicated.

3.6 CELL VIABILITY ASSAY

Cell viability was assessed as previously described ([275,276]) by employing the ATPlite kit (Promega, Milano, Italy) and luminescent output (relative luminescence units, RLU) was recorded by the EnVision™ 2104 Multilabel Reader (PerkinElmer Life Sciences, Boston, MA, USA). The CellTiter-Glo® Luminescent Cell Viability Assay is a homogeneous method of determining the number of viable cells in culture based on quantification of the ATP present, an indicator of metabolically active cells. The Assay relies on the properties of a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase), which generates a stable "glow-type" luminescent signal caused by the reaction of ATP with luciferase and D-luciferine, as shown in Figure 12, with a consequent light emission, which is proportional to the ATP concentration and in turn, is directly proportional to the cells number present in the culture.

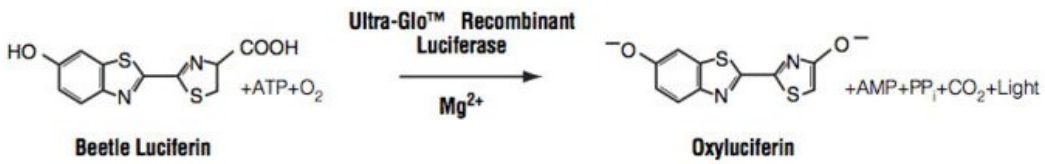

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Figure 12: *The luciferase reaction. Mono-oxygenation of luciferin is catalysed by luciferase in the presence of Mg2+, ATP and molecular oxygen.*

Briefly, the cells were seeded at 2 x 10^4/well in 96-well black plates and treated with TGF-β 1 pM. Control cells were treated with the vehicle alone (0.1% DMSO); treatments were renewed every 24 h. Concerning the primary cultures, the cells were seeded at 1 x 10^4/well in 96-well black plates and treated with Everolimus and/or IGF-1 100 nM, respectively. After incubation time (48 h for primary cultures and 72h for cell lines), cell viability assay was assessed adding substrate solution directly to cell culture plates. Results are expressed as mean value ± standard error of the mean (S.E.M) percent RLU vs. untreated control cells in six replicates.

3.7 CASPASE ACTIVATION ASSESSMENT

Caspase activation was evaluated by using the Caspase-Glo 3/7 assay (Promega, Milano, Italy) according to the manufacture's instructions ([277]). Luminescent signal was measured with the EnVision™ 2104 Multilabel Reader and expressed as relative luminescence units (RLU). The Caspase-Glo® 3/7 Assay is a homogeneous, luminescent assay that measures caspase-3 and -7 activities. These members of the cysteine aspartic acid-specific protease (caspase) family play key effector roles in apoptosis in mammalian cells. The Caspase-Glo® 3/7 Reagent relies on the properties of a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase), which is formulated to generate a stable "glow-type" luminescent signal, after cell lysis and caspase cleavage of the substrate. The light emitted is directly proportional to the amount of the caspase activity present (Figure 13).

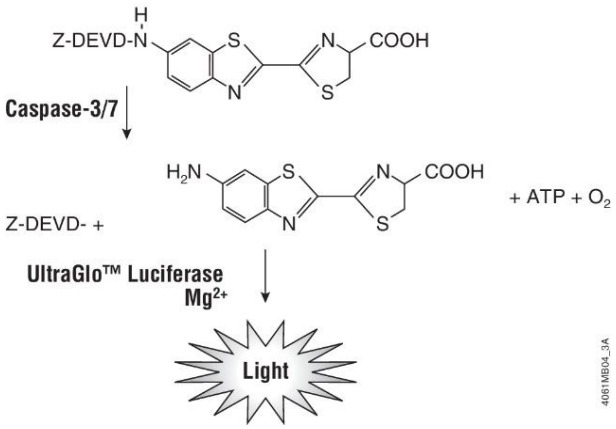


Figure 13: *Caspase 3/7 cleavage of the luminogenic substrate containing the DEVD sequence. Following caspase cleavage, a substrate for luciferase (aminoluciferin) is released, resulting in the luciferase reaction ant the production of light.*

Briefly, the cells were seeded, treated and incubated with the indicated compounds as previously described for the CellTiter-Glo® Luminescent Cell Viability Assay on both primary cultures and *in vitro* cell lines. Results are expressed as mean value ± S.E.M percent RLU vs. untreated control cells in six replicates.

3.8 TRANSWELL MIGRATION ASSAY

The transwell migration assay, also called Boyden chamber assay, was used to assess the ability of cells to migrate. The principle of this assay is based on two medium containing chambers separated by a porous membrane (8.0 μm) through which cells transmigrate. Briefly, the cells, under confluence status, were seeded in RPMI 1640 medium (2% FBS) in the upper part of the chamber, at $5x10^4$/0.2 ml/well (0.25 x 10^6 cells/ml) in 24-well plates. The cells migrated in vertical direction through the pores of the membrane into the lower compartment, in which medium with higher serum content (20% FBS) was present. After 1 h, the cells that passed the membrane were fixed on the membrane, stained and quantified. While non-migrated cells remaining on the topside of the filter were removed with a cotton swab, the migrated cells were stained with cytological dyes (haematoxylin and eosin) and the number of stained cells was determined visually by counting them with Eclipse TE2000 Inverted Microscope (Nikon Instruments S.p.A, Firenze, Italy). Results are expressed as mean value ± S.E.M of two independent experiments.

3.9 PROTEIN ISOLATION

The cells were seeded at a cell density of 2 x 10^4 cells/ml in 75-mm plates in complete medium (10% FBS). Cells were synchronized by overnight incubation in 0.1% FBS medium, and the day after, cells were treated with or without TGF-β1 in complete medium and than after 24 h, cell pellets were collected. For protein isolation from human frozen tissues, total cell lysates were obtained by using Tissue Raptor (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Human cell lines and human tissues were dissolved in RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA) (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS), kept in ice for 30 min, and then centrifuged at 13,000 rpm for 20 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).

3.10 WESTERN BLOT ANALYSIS

For protein evaluation, proteins were mixed with sample buffer (60 mM Tris-HCl (pH 6.8), 10% glycerol, 2% sodium dodecyl sulphate) and lysed by boiling at 95°C for 5 min. Subsequently, 30 μg of lysates were fractionated on 10% SDS-PAGE and transferred by electrophoresis to polyvinylidene difluoride (PVDF) membranes (PerkinElmer) by using the Lightning BlotterTM (PerkinElmer). The membranes were incubated with the following antibodies: polyclonal rabbit anti-human TSC22D1 (Abcam, Cambridge, UK), monoclonal rabbit anti-human TGF-β (Cell Signaling Technology, Beverly, MA, USA), polyclonal rabbit anti-human TβR-I (Cell Signaling Technology), monoclonal rabbit anti-human TβR-II (Cell Signaling Technology), polyclonal rabbit anti-human Smad 2/3 (Cell Signaling Technology), polyclonal rabbit anti-human Smad 4 (Cell Signaling Technology), polyclonal rabbit anti-human Smad 1 (Cell Signaling Technology), monoclonal mouse anti-human Smad 6 (Thermo Scientific Inc), polyclonal rabbit anti-human Importin-β1 (Cell Signaling Technology), monoclonal mouse anti-human E-cadherin (Abcam), polyclonal rabbit anti-human Caspase-3 (Cell Signaling Technology), monoclonal rabbit anti-human GAPDH (Cell Signaling Technology). All the antibodies were diluted at 1:1000. Anti-rabbit or anti-mouse HRP IgG antibodies (Dako Italia, Milano, Italy) were used at a dilution of 1:5000, and bindings was revealed using the Pierce™ ECL Western Blotting and SuperSignal™ West Femto Maximum Sensitivity Substrates (Thermo Scientific). Quantification of the band intensity was performed by using a Gel Doc System, with the Quantity One Software (Bio-Rad, Hercules, CA, USA). Three independent experiments were performed; data are expressed as the ratio between protein of interest and GAPDH signal intensity, expressed as percentage vs. control.

3.11 KINASE ACTIVITY ASSAY

The Amplified Luminescent Proximity Homogeneous Assay (PerkinElmer Life Sciences) was used to measure the phosphorylated levels of IGF1R (Tyr1135/1136), AKT (Ser473), mTOR (Ser2448), 4EBP1 (Thr37/46). The AlphaScreen SureFire (Figure 14) 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. 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.

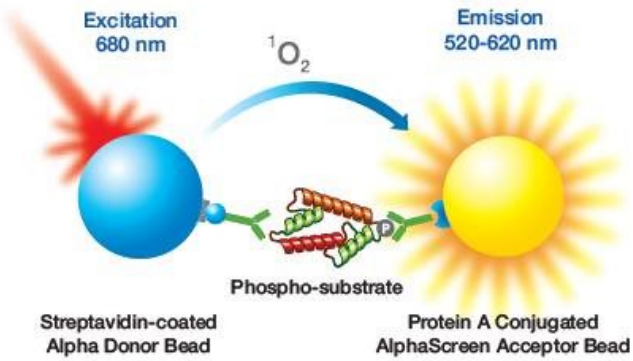


Figure 14: *AlphaScreen SureFire Assay Principle*

Briefly, the expression levels of the phosphorylated proteins were evaluated using proteins isolated from frozen tissues. For each sample, 3-6 replicates were assessed, using the same total protein amount into a 384-well culture plate. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was employed for normalization. Results are expressed as mean value ± S.E.M. AlphaScreen signal (counts).

3.12 TISSUE MICROARRAY CONSTRUCTION

Tissue microarrays (TMA) were manually constructed using formalin-fixed, paraffin-embedded tissues. TMAs are produced by the repeated transfer of small tissue cores, from paraffin embedded "donor" blocks into a single TMA "recipient" block; this repeated transfer of tissue cores leads to a construction of a "tissue archive" that can contain hundreds of tissue samples from a small or large number of patients ([278]). From each block, three 1 mm-diameter normal and tumoral tissue cores were taken. Multiple 3-µm sections were cut with a Leica microtome (Leica Microsystems Inc, Bannockburn, IL, USA), dried at 60°C and then transferred to adhesive-coated slides for immunohistochemical staining.

3.13 IMMUNOHISTOCHEMISTRY

For the IHC, slides were de-paraffinized and re-hydrated in dewax solution (Leica Biosystems, Newcastle, UK) and H_2O_2 was used for 4 min to block the endogenous peroxidase activity. The antigen retriavel was performed using buffer citrate pH 6.0 (15 min at 100°C), and subsequently slides were incubated with a monoclonal rabbit anti-human phospho (Ser 463) AKT (Abcam) antibody (1:300) for 30 minutes, at room temperature. The Bond Polymer Refine Kit on the automated system BOND RX (both from Leica Biosystems) was employed to reveal the immunoreactions. The detection of antigen-antibody complexes was assessed using the chromogen cobalt-3,3-diaminobenzidine (Co-DAB) (Leica Biosystems). The chromogen reacts with HRP to form a brown precipitate at the site of antibody binding. Samples were counterstained with haematoxylin and subsequently mounted in Aquatex (Merck, Darmstadt, Germany).
Parallel control sections, in which the primary antibodies were omitted, were used to verify the specificity of reactions. Endometrial cancer tissues were employed as positive controls, since a PTEN mutation leading to PI3K/AKT pathway constitutive activation characterises the majority of them. Tumor staining characteristics were reviewed and interpreted independently by two pathologists (AP and DN). IHC data were visually scored. The immunostaining intensity was evaluated subjectively as negative (0), weak (1), moderate (2) and strong (3) and then the staining was classified into two categories: negative (no immunostaining) and positive (weak-moderate and strong immunostaining), in more than 10% of tumor cells.

3.14 STATISTICAL ANALYSIS

Results are expressed as the mean ± S.E.M. A preliminary analysis was carried out to determine whether the datasets conformed to a normal distribution. The results were compared within each group and between the groups using ANOVA. If the F values were significant ($P<0.05$), Student's paired or unpaired t-test was used to evaluate individual differences between the means. P values <0.05 were considered significant. In case of not normal distribution, Mann-Whitney test was used. To compare clinical-pathological features, Chi Square and Fisher's exact tests were used. Data were analysed using GraphPad (Prism v-6.0).

4. RESULTS

4.1 PART I

4.1.1 EFFECT OF EVEROLIMUS AND IGF-1 ON CELL VIABILITY OF P-NET PRIMARY CULTURES

20 P-NETs primary cultures were incubated for 48 h in a culture medium supplemented with 100 nM Everolimus alone (green bars) or in combination with 100 nM IGF-1 (grey bars). The responsiveness to the mTOR inhibitor has been evaluated in terms of cell viability reduction. We defined as *responders* (P-NET-R) those primary cultures displaying a significant reduction ($P<0.05$ vs. untreated cells) in cell viability under Everolimus treatment; on the contrary, we defined as *non-responders* (P-NET-NR) those primary cultures in which Everolimus did not reduce cell viability. On this basis, six P-NET primary cultures were classified as P-NET-R and 14 P-NETs were classified as P-NET-NR. Figure 15A shows that, cell viability was significantly reduced by Everolimus (-29.9%; $P<0.05$ vs. untreated cells), in P-NET-R, an effect completely counteracted by co-incubation with IGF-1. On the contrary, we observed that in P-NET-NR Everolimus significantly induced cell viability (+ 30.2 %; $P< 0.001$ vs. control cells), and IGF-1 did not affect this parameter, neither alone nor in combination with Everolimus (Figure 15B).

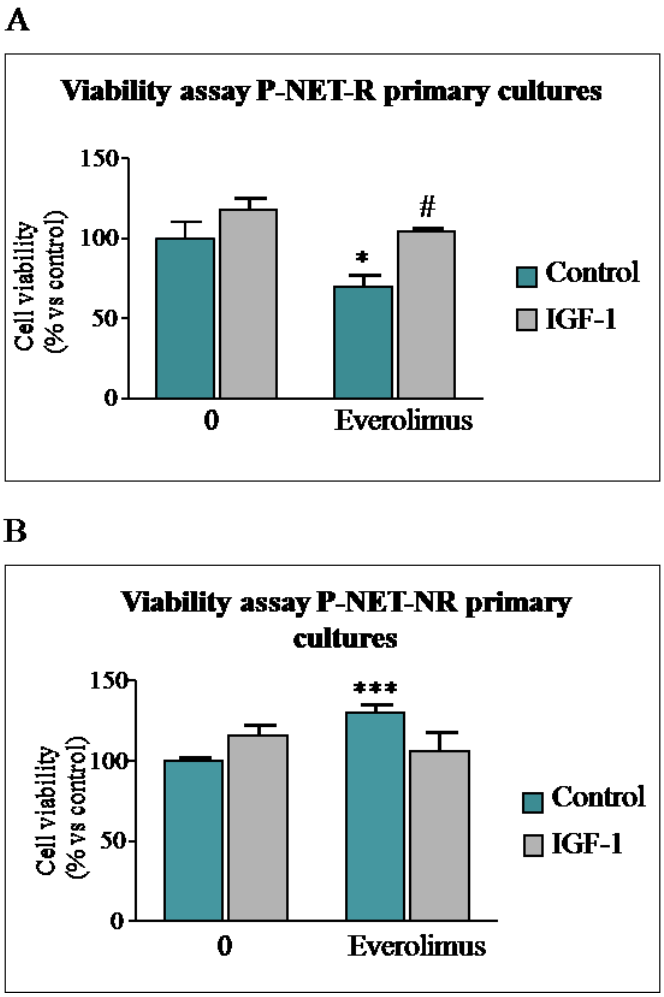


Figure 15: *Cell viability assay of P-NET R (A) and P-NET-NR (B) primary cultures. * P<0.05 and ***P<0.001 vs. untreated control cells. #P<0.05 vs. cells treated with Everolimus alone.*

Subsequently, caspase 3/7 activation was evaluated in the primary cultures treated with the previous compounds to verify whether the effects of Everolimus on cell viability were determined by an influence on the apoptotic process. We found that caspase activation was significantly induced by Everolimus treatment (+73%; P<0.001 vs. untreated cells) in P-NET-R (Figure 16A); co-incubation with IGF-1 completely counteracted this effect. On the contrary we observed that caspase activation was not significantly affected both by Everolimus and IGF-1 in P-NET-NR (Figure 16B). Our data indicate that the responsiveness to Everolimus in vitro may be different in P-NET and that the mechanism of resistance to Everolimus in vitro may due to an inactive IGF-1 signalling pathway.

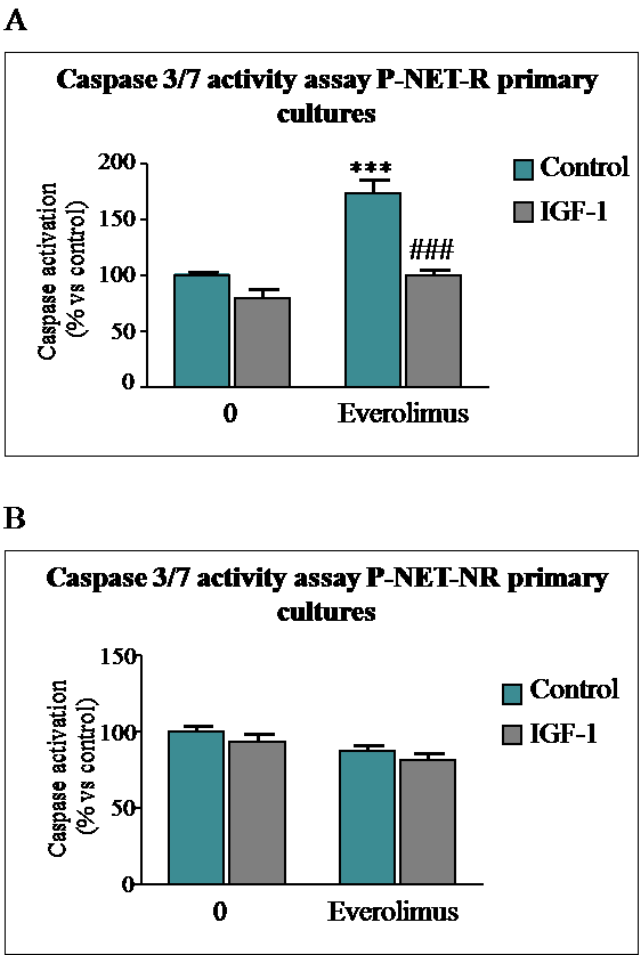


Figure 16: *Caspase 3/7 activity assay of P-NET R (A) and P-NET-NR (B) primary cultures. ***P<0.001 vs. untreated control cells. ###P<0.001 vs. cells treated with Everolimus alone.*

4.1.2 IGF1/mTOR SIGNALLING PATHWAY EXPRESSION IN P-NET TISSUES

Since the effect of Everolimus on cell viability was influenced by IGF-1 only in human P-NET tissues classified as responders to the drug in vitro, but not in human P-NET tissues resistant to the drug, we investigated the levels of IGF-1 downstream signalling proteins involved in mTOR pathway aimed at verifying whether IGF-1 pathway is involved in the mechanism of resistance to Everolimus. Total proteins were isolated from frozen P-NET tissues, classified as *responders* and *non-responders*, on the basis of the results of primary cultures, and AlphaScreen analysis for p-IGF-1R, p-AKT, p-mTOR and p-4EBP1 expression in the pooled P-NET-R and in the pooled P-NET-NR tissues was performed.

Figure 17A showed that p-IGF-1R protein levels were significantly (P<0.001) >2-fold higher in P-NET-R as compared to P-NET-NR. p-AKT levels were >2-fold higher in P-NET-R than to P-NET-R (Figure 17B); however, possibly because of the small number of samples, statistical significance was not reached. Similarly, p-mTOR and p-4EBP1 protein levels were significantly (P<0.02 and P<0.001) >2-fold higher in P-NET-R as compared to P-NET-NR (Figure 17C and Figure 17D). These data indicate an association between the response to Everolimus in vitro and an active AKT/mTOR pathway.

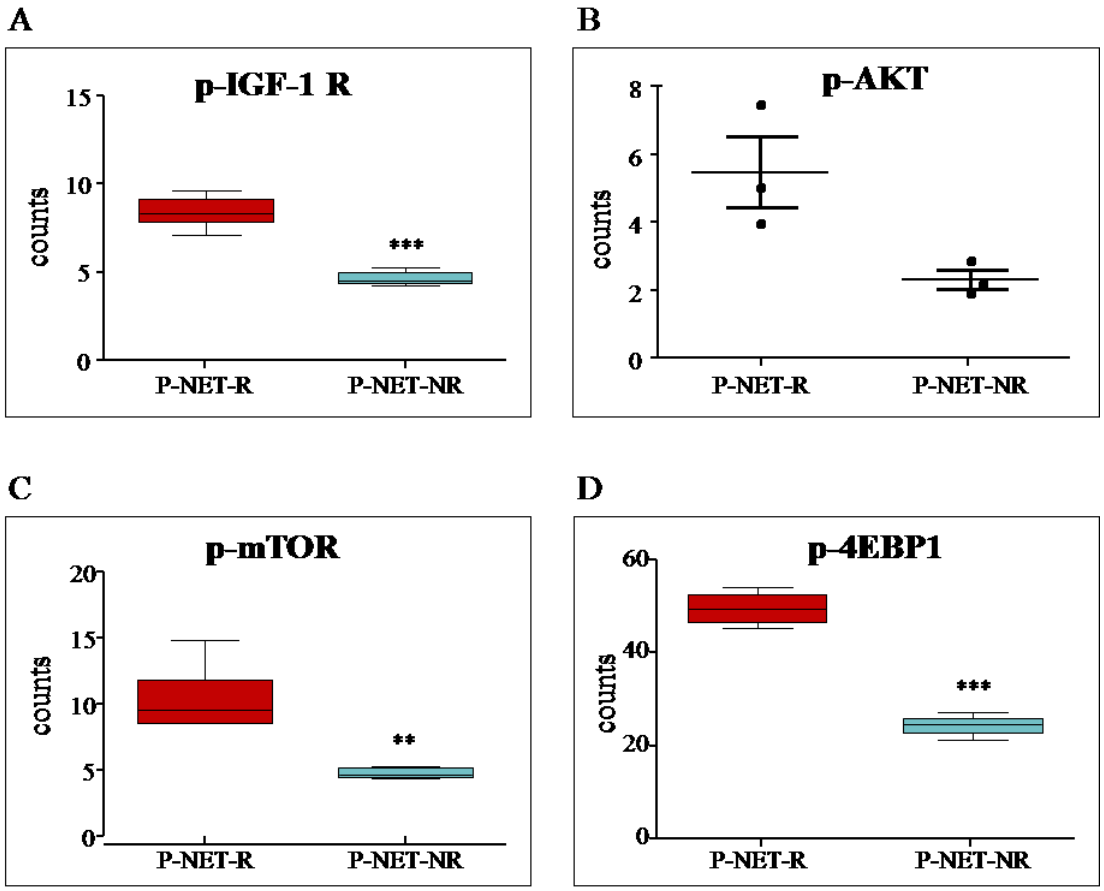


Figure 17: *AlphaScreen Surefire Assay for p-IGF-1R (A), p-AKT (B), p-mTOR (C) and p-4EBP1 (D) expression on P-NET tissues. ** P<0.02 and ***P<0.001 vs. P-NET R.*

4.1.3 CLINICAL AND PATHOLOGICAL CORRELATIONS

In addition, we also investigated whether could be some differences in terms of clinical characteristics between P-NET-R and P-NET-NR. Therefore, the clinical and pathological features of the patients were evaluated according to Everolimus responsiveness in vitro. We found no significant difference between P-NET-R and P-NET-NR patients in terms of

sex, age, primary vs. metastatic tissue, pre-treatment with SSA, Octreoscan/68GA-PET uptake, site, TNM, stage, size, number of mitoses, necrosis. However ki-67 was significantly ($P<0.05$) higher in P-NET-R (median 10%) than in P-NET-NR (median 2%) (Figure 18A). Furthermore, we found a trend for a linear correlation between tumor grade and response to Everolimus; in particular we observed that all G3 P-NET responded to the treatment in vitro, while only 14% of G1 P-NET were P-NET-R (Figure 18B).

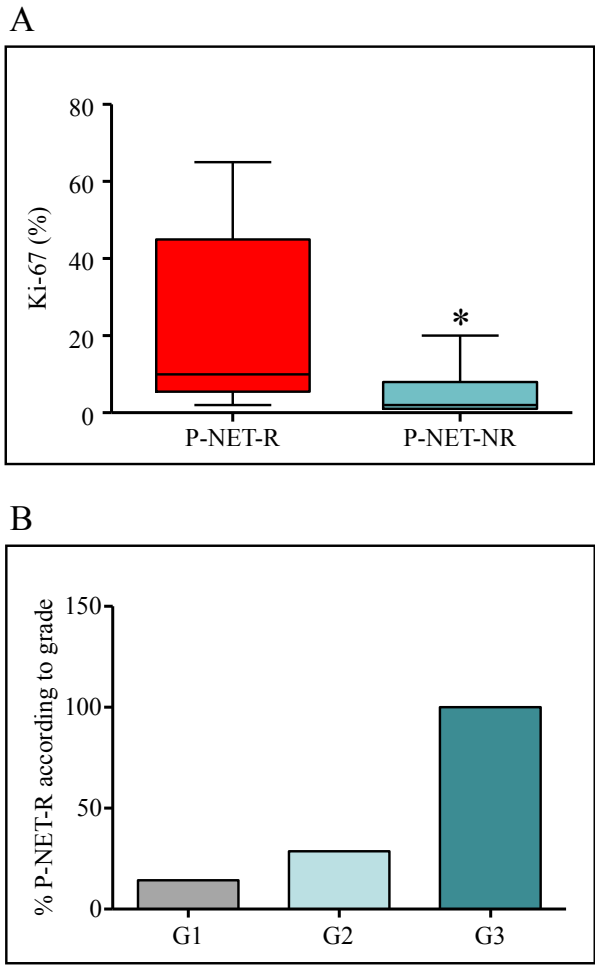


Figure 18: *Patients clinical characteristics and Everolimus responsiveness in vitro. (A) Median values and range of Ki67 labelling index in P-NET-R and in P-NET-NR. *P<0.05 % vs. P-NET-R. (B) % P-NET-R tissues according to tumor grade. G1 P-NET-R= 1/7; G2 P-NET-R =25 2/7; G3 P-NET-R= 2/2.*

4.1.4 EXPRESSION OF mTOR PATHWAY COMPONENTS IN P-NET AND ASSOCIATION WITH CLINICAL OUTCOMES

To validate the previous results, we assessed IHC for p-AKT protein on P-NET tissues. We obtained reliable results only for 11 patients, 3 P-NET-R and 8 P-NET-NR, due to technical problems. We found that p-AKT IHC staining was positive in all the paraffin embedded tissues classified as P-NET-R in vitro; on the other hand we found that p-AKT staining was positive in 5 out of 8 paraffin embedded tissues classified as P-NET-NR in vitro. However, we did not find a statistically significant association between p-AKT positivity by IHC and responsiveness to Everolimus in vitro, probably due to the low number of available samples. Subsequently, the Everolimus responsiveness in vitro, and p-AKT protein levels assessed by IHC were also compared with the response to Everolimus in vivo, in terms of tumor stabilization/progression and survival. Among 16 patients, two had been treated with Everolimus after surgery for persistence of disease due to the presence of unresectable liver metastases. The first patient was a 56 years old male operated on for a well differentiated non functioning G3 P-NET (T3N1M1, Ki67=25%), whose primary culture was *responder* to Everolimus in vitro. The patient displayed stabilization of the disease during Everolimus treatment, with a progression free survival of 21 months (still alive at the end of the study). Furthermore, his IHC score was positive for p-AKT at tissue level (Figure 19A). The second patient was a 65 years old female operated on for a non functioning G2 P-NET (T3N1M1, Ki67= 20%), whose primary culture was *non-responder* to Everolimus in vitro. The patient displayed disease progression after 12 months of treatment with Everolimus, with a survival of 21 months (still alive at the end of the study). Her IHC score was negative for p-AKT (Figure 19B).

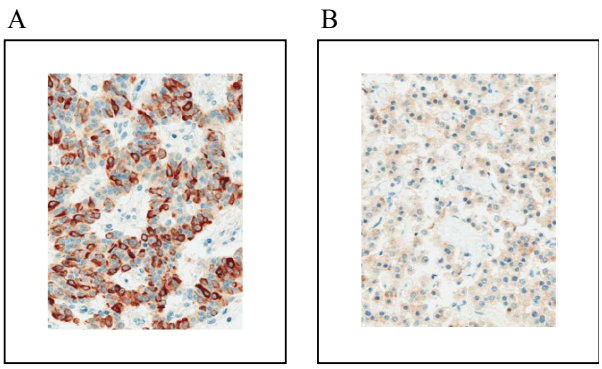


Figure 19: *Immunohistochemical expression of p-AKT in a P-NET sensitive to Everolimus in vitro and defined as P-NET-R in vivo (A) and in a P-NET non-responder to Everolimus in vivo and defined as P-NET-NR in vitro (B).*

4.2 PART II

4.2.1 TSC22D1 EXPRESSION IN HUMAN BCs

Twenty human BC tissues were characterized for TSC22D1 expression by Western blot analysis. As shown in Figure 20, TSC22D1 protein levels were detectable in 10 out of 20 BCs (50 %) samples.

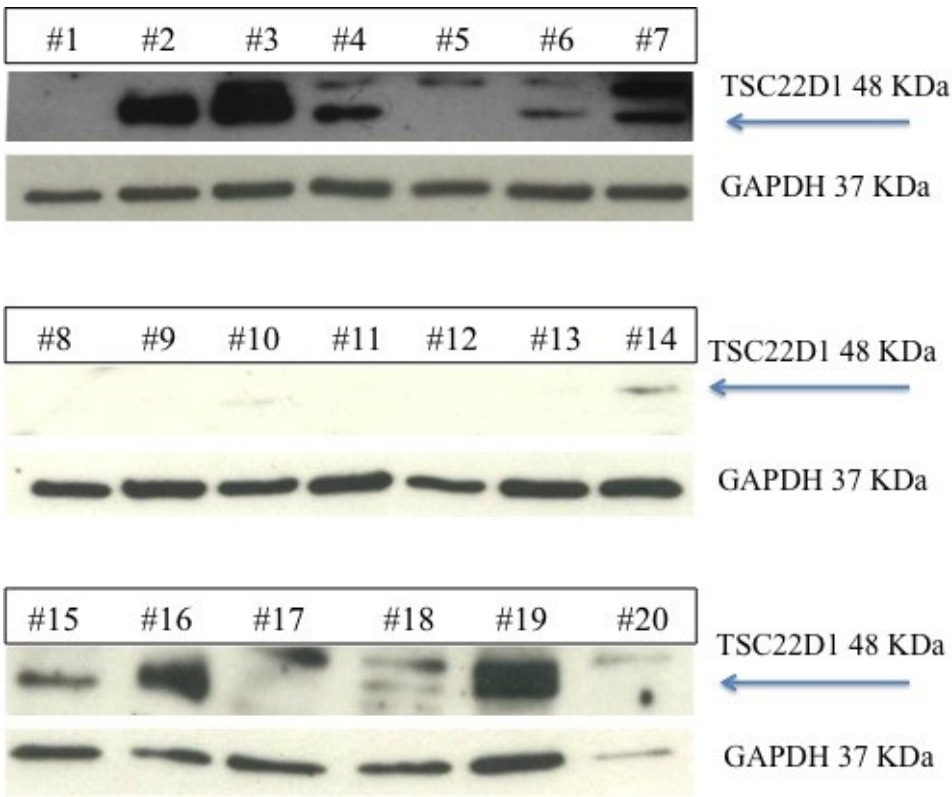


Figure 20: *TSC22D1 protein expression in human BC tissues.*

4.2.2 TSC22D1 GENE SILENCING IN A HUMAN BC CELL LINE

To evaluate the functional role of TSC22D1 gene, the effects of down-expression were considered. NCI-H727 cells (Figure 21A) were stable transfected with a shTSC22D1 plasmid, and after treatment with puromycin, a clone was selected, referred as sh-NCI-H727 (Figure 21B). The efficiency of transfection was assessed, by analysing TSC22D1 expression using Western blot. As shown in figure 21C, TSC22D1 protein expression was reduced in cells transfected with shTSC22D1 plasmid as compared to untransfected-control cells. NCI-H720 cells were used as negative control. Subsequently, protein levels were quantified by densitometry analysis (Figure 21D); data showed a decrease in TSC22D1 protein expression by 42.7% in sh-NCI-H727 cells compared to control cells, after normalization to GAPDH levels.

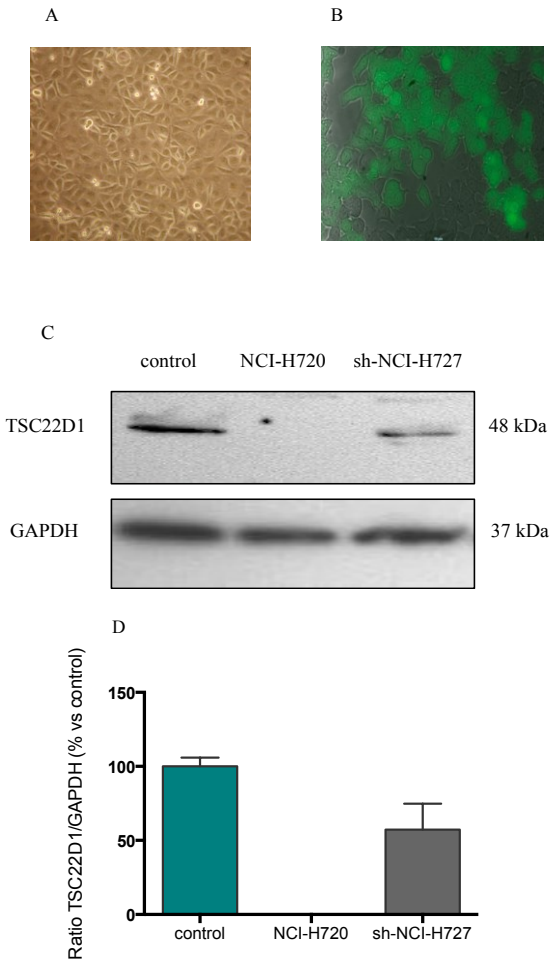


Figure 21: *(A) NCI-H727 cells. (B) sh-NCI-H727 cells. (C) Western blot analysis for TSC22D1 expression in NCI-H727 (positive control cells) NCI-H720 (negative control cells) and sh-NCI-H727 (TSC22D1 silenced cells). GAPDH is shown as a loading control. (D) Densitometry analysis of TSC22D1 protein levels. Data are shown as the mean ± SEM of three independent experiments.*

4.2.3 EFFECT OF TSC22D1 SILENCING ON CELL MIGRATION

In order to understand whether TSC22D1 affects the ability of BC cells to migrate, we evaluated cell motility of each cell line, by assessing a Transwell migration assay. We found that cells successfully penetrated through the porous membrane-coated chambers, however, sh-NCI-H727 cells showed lower ability to migrate (-42%) as compared to control cells (Figure 23A). To determine the potential mechanism by which TSC22D1 silencing decreases the BC cells motility, E-cadherin protein expression levels were assessed in control and sh-NCI-H727 cells by Western blot analysis (Figure 23B). Sh-NCI-H727 cells displayed higher E-cadherin levels (+67%) as compared to control cells. These data indicate that TSC22D1plays an important role in the capacity for motility in BC cells, and its silencing may determine an increase in E-cadherin protein levels, resulting in increased cell-to-cell interactions, and possibly explaining the decreased migration ability.

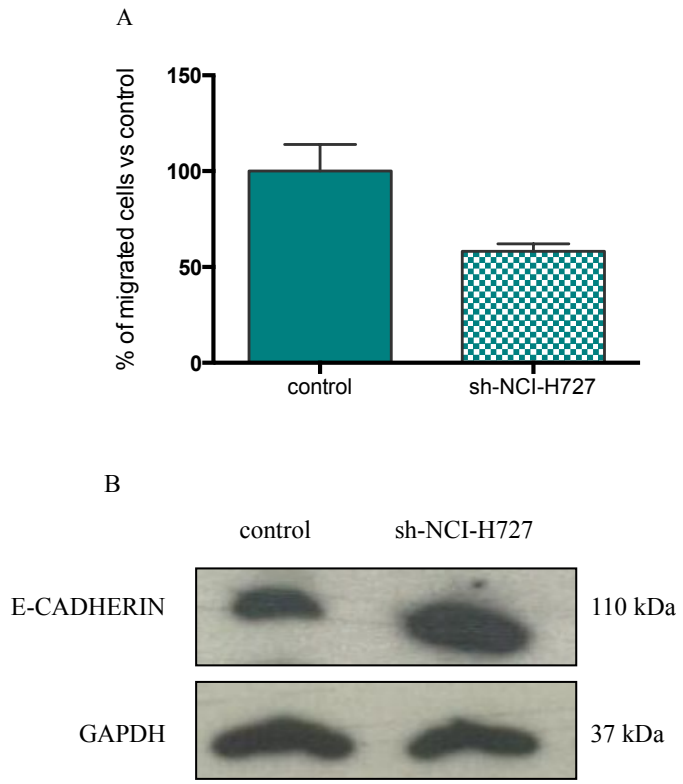


Figure 22: *(A) Transwell migration assay. The graph represents the percentage of migrated cells after 1 h vs. control. (B) Western blot analysis for E-cadherin expression in control cells and in sh-NCI-H727 cells. GAPDH is shown as a loading control.*

4.2.4 EFFECTS OF TGF-β ON CELL VIABILITY AND APOPTOSIS OF BC CELLS

To determine whether TSC22D1 influences the effects of TGF-β on cell viability of BC cells, control and sh-NCI-H727 cells were incubated for 72 h in a culture medium supplemented with 1 pM TGF-β. As shown in Figure 22A, TGF-β significantly reduced cell viability (-66.7%; P< 0.02 vs. untreated cells) in NCI-H727 cells. On the contrary, we observed that the viability was not affected by TGF-β in sh-NCI-H727 cells. In order to verify whether TSC22D1 influences the effects of TGF-β on the apoptotic mechanisms, caspase 3/7 activation was evaluated. As shown in figure 22B, TGF-β determined an increase in apoptosis in NCI-H727 cells, but it did not affect this parameter in sh-NCI-H727 cells. These data indicate that TSC22D1 may play a crucial role in affecting the anti-proliferative and pro-apoptotic effects induced by TGF-β.

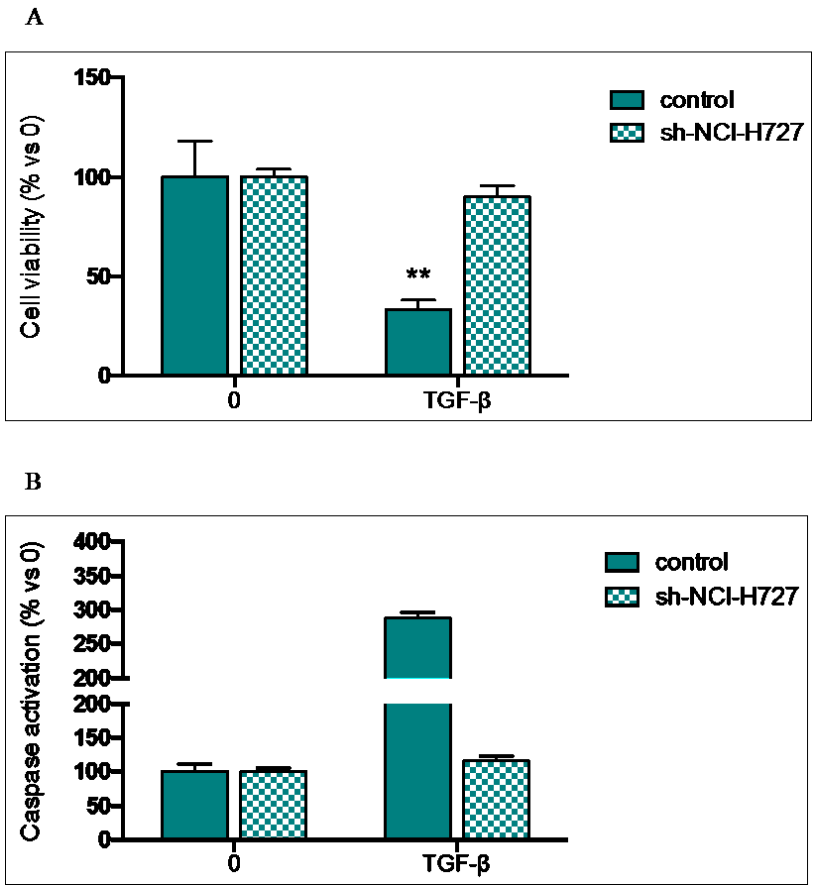


Figure 23: *Cell viability assay (A) and Caspase 3/7 activity assay (B) of control cells and sh-NCI-H727 cells. ** P<0.02 vs. untreated cells.*

4.2.5 ROLE OF TSC22D1 IN TGF-β SIGNALLING PATHWAY

Since we observed that TSC22D1 silencing affects the effects of TGF-β on cell viability in BC cells, we evaluated the expression levels of TGF-β downstream signalling proteins in control cells and sh-NCI-H727 cells, in order to understand whether TSC22D1 influences the TGF-β activity, by modifying protein profile of BC cells. Western Blot analyses for TGF-β, TβR-I, TβR-II, Smad 2, Smad 3, Smad 4, Smad 1, and Smad 6 expression were performed. As shown in Figure 24, no difference was found in TGF-β and TβR-I protein levels between control and sh-NCI-H727 cells. On the other hand, lower levels of TβR-II protein (≈ -20%) were found in sh-NCI-H727 cells as compared to control cells.

In addition, Figure 25 shows that both Smad 2, Smad 3, Smad 4, Smad 1 protein levels were higher in sh-NCI-H727 cells (+32%, +36%, +75%, +36%, respectively) than in control cells. Similarly we found that Smad 6 protein levels were higher in sh-NCI-H727 as compared in control cells, where the protein expression was undetectable. These data indicate TSC22D1 is involved in TGF-β signalling pathway regulation.

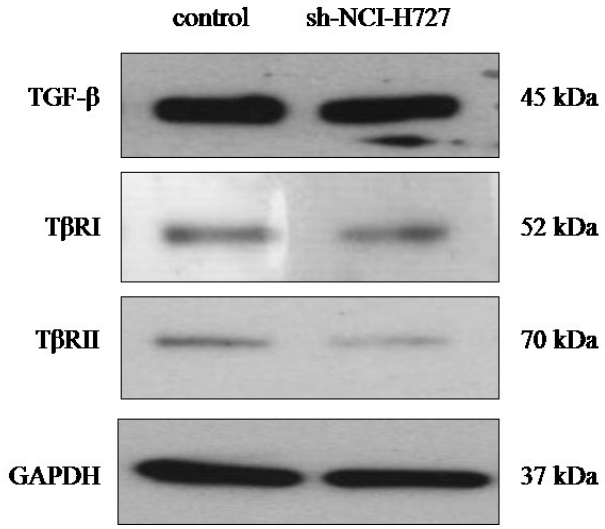


Figure 24: *Western Blot analyses for TGF-β, TβR-I, TβR-II expression in control cells and in sh-NCI-H727 cells. GAPDH is shown as a loading control.*

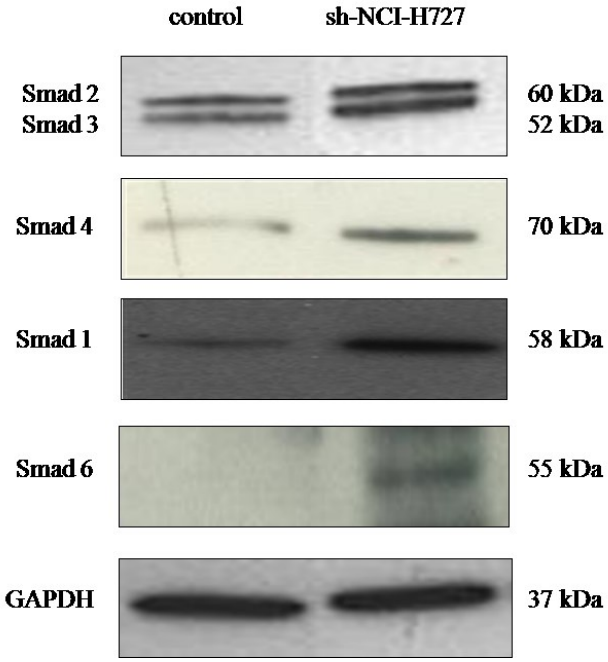


Figure 25: *Western blot analyses for Smad proteins expression involved in TGF-β signalling pathway in control cells and in sh-NCI-H727 cells. GAPDH is shown as a loading control.*

Subsequently, we investigated whether TGF-β treatment was able to influence the expression levels of TGF-β pathway components. Therefore, Smad 2, Smad 3, Smad 4 and Importin-β expression was evaluated at basaline level and after treatment with 1 pM TGF-β by Western blot analysis. As shown in Figure 26, in keeping with the results shown in Figure 25, Smad 2, Smad 3, and Smad 4 basal levels were higher in sh-NCI-H727 cells as compared to control cells. In addition, we observed that basal Smad 2 and Smad 3 levels were not influenced by treatment with TGF-β in sh-NCI-H727 cells, but TGF-β slightly increased Smad 3 protein expression, and slightly decreased Smad 2 protein levels in control cells. Similarly, we found that in sh-NCI-H727 cells, the basaline Smad 4 level was not affected by TGF-β treatment; on the contrary Smad 4 expression was increased (+35.5%) by treatment with TGF-β in control cells. Furthermore, no difference in Importin-β basaline levels was found between control and sh-NCI-H727 cells; on the other hand we observed that Importin-β expression was increased by TGF-β treatment in control cells (+45.7%); its expression was not affected by TGF-β treatment in sh-NCI-H727 cells.

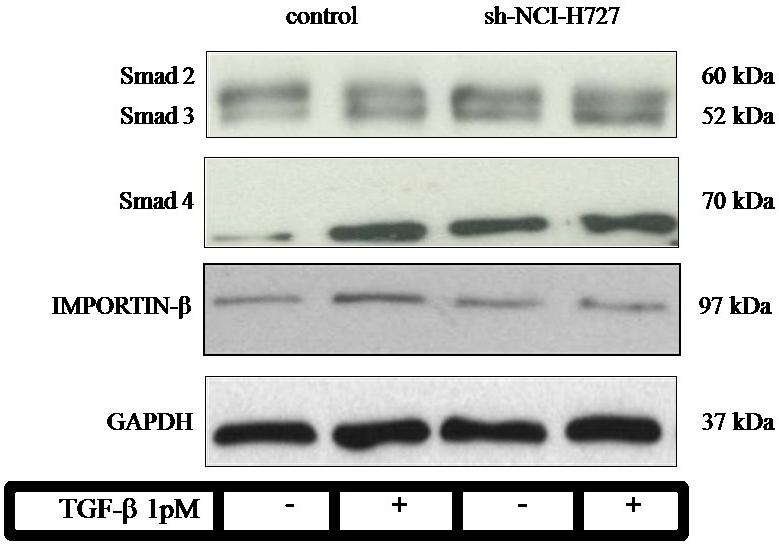


Figure 26: *Effects of TGF-β treatment on Smad 2, Smad 3, Smad 4 and Importin-β expression in control and sh-NCI-H727 cells. GAPDH is shown as a loading control.*

To better understand the mechanism by which TSC22D1 affects the pro-apoptotic effects of TGF-β in BC cells, we analysed the Caspase 3 protein expression by Western blot, both at baseline level and after treatment with TGF-β, in control cells and in sh-NCI-H727 cells. As shown in Figure 27A, sh-NCI-H727 cells displayed lower total and cleaved caspase 3 protein levels (-63%) as compared to control cells, confirming what was previously measured by caspase 3/7 activation. In addition, we found that TGF-β treatment induced an increase in cleaved caspase 3 protein levels (+71%) in sh-NCI-H727 cells, effect not observed in control cells (Figure 27B).

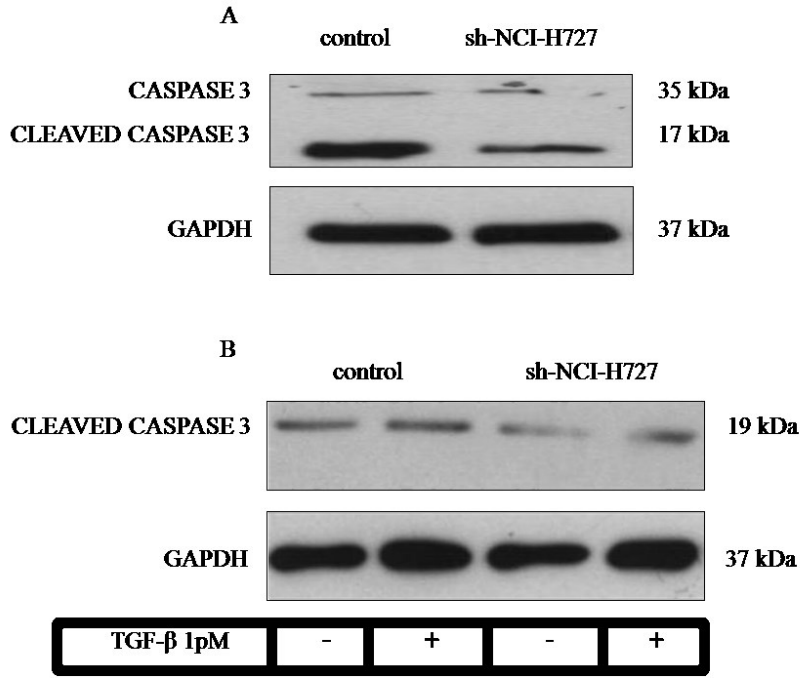
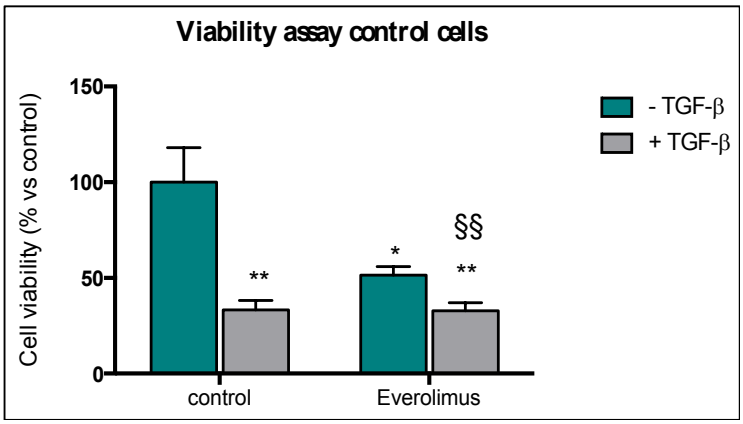


Figure 27: *(A) Western blot analysis for total and cleaved caspase 3 protein expression at basaline level in control and sh-NCI-H727 cells. (B) Effects of TGF-β treatment on cleaved caspase 3 expression in control and sh-NCI-H727 cells. GAPDH is shown as a loading control.*

4.2.6 COMBINED EFFECTS OF TGF-β AND EVEROLIMUS ON CELL VIABILITY AND APOPTOSIS IN BC CELLS

In order to evaluate whether TSC22D1 modulate the effects of Everolimus in BC cells, control and sh-NCI-H727 cells were treated with 100 nM Everolimus alone (green bars) or in combination with 1 pM TGF-β (grey bars). After 72 h treatment, cell viability and caspase 3/7 activation were evaluated. As shown in Fig. 28A, in control cells, Everolimus, as single agent, significantly reduced cell viability (-48.6%; $P<0.05$ vs. untreated cells); and TGF-β significantly reduced this parameter either alone (-66.7%; $P< 0.02$ vs. untreated cells), or in combination with Everolimus (-67.2%; $P< 0.02$ vs. untreated cells). Therefore we observed that Everolimus did not affect TGF-β effects on cell viability. Concerning the apoptotic process, a concomitant increase in apoptosis activation was observed in control cells after the same treatments (Fig. 28B).

A



B

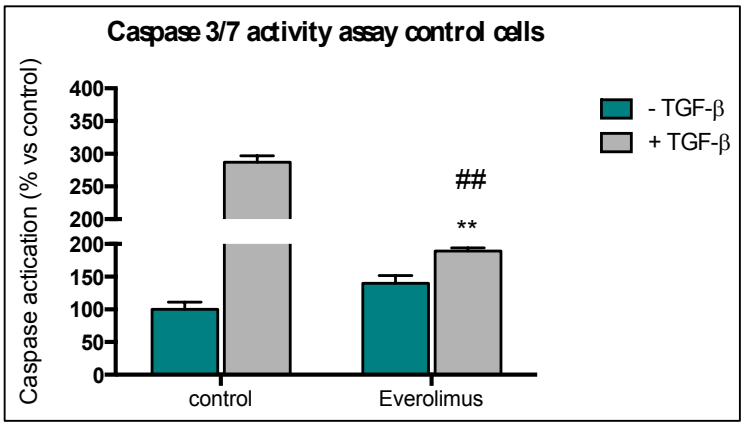


Figure 28: *Cell viability assay (A) and Caspase 3/7 activity assay of control cells. * P<0.05 and **P<0.02 vs. untreated control cells; §§ P<0.01 vs. cells treated with Everolimus alone; ## P<0.01 vs. cells treated withTGF-β alone.*

On the other hand, as shown in Figure 29A, we found that in sh-NCI-H727 cells, Everolimus significantly reduced cell viability (-28%; *** P<0.001 vs. untreated cells), and TGF-β, as single agent, did not affect this parameter. Furthermore, we found that, TGF-β did not increase the anti-proliferative effects of Everolimus (-32.6%; *** P<0.001 vs. untreated cells). These data confirm that TSC22D1 silencing abolished the anti-proliferative effects of TGF-β. In addition, our data suggest that TSC22D1 may be an important mediator of Everolimus effects in BC cells, since Everolimus exerted a lower reduction on cell viability in silenced cells as compared to control cells. Concerning the apoptotic process, no significant effects were observed after the same treatments in sh-NCI-H727 cells (Fig. 29B).

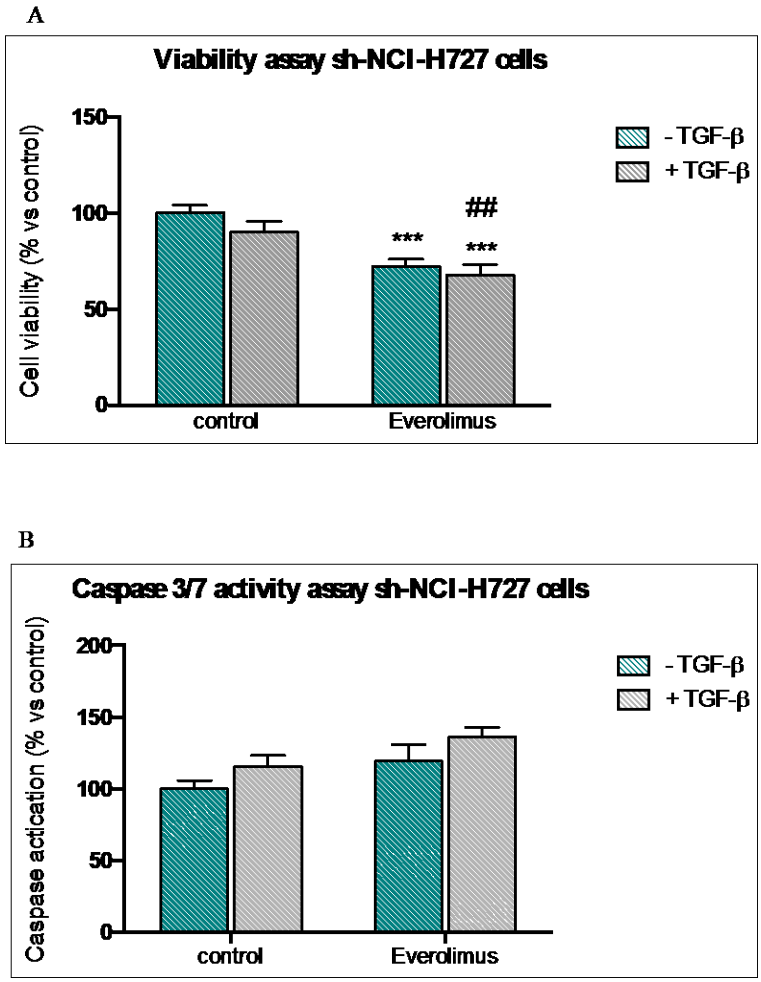
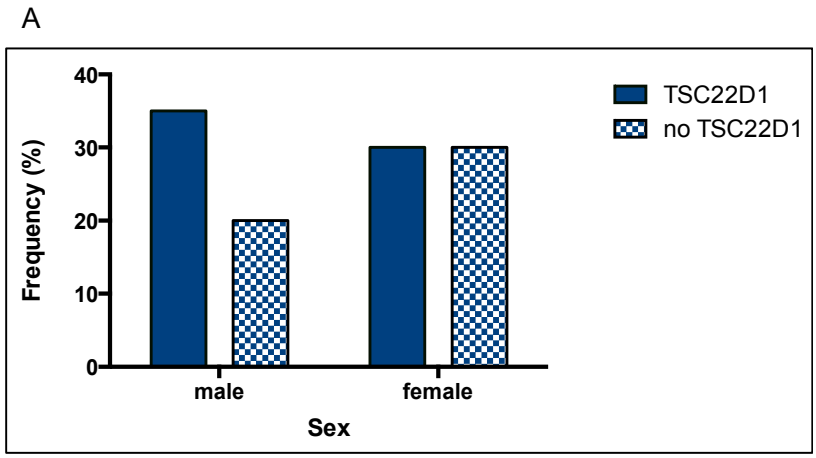


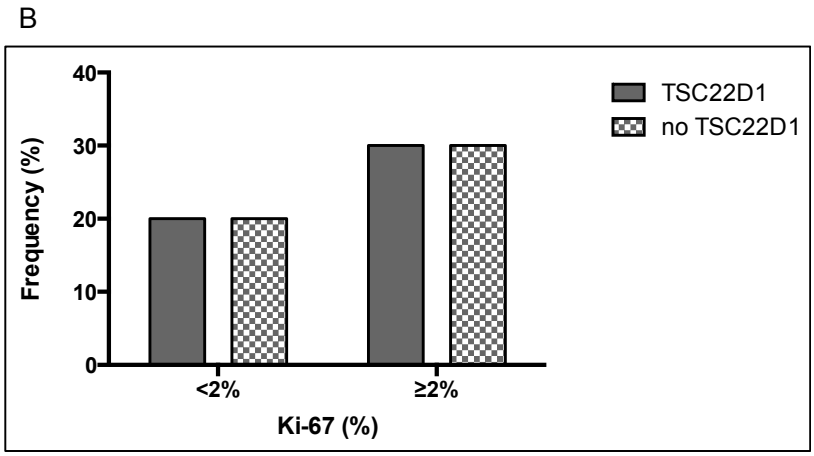
Figure 29: *Cell viability assay (A) and Caspase 3/7 activity assay of sh-NCI-H727 cells. *** P<0.001 vs untreated cells; 0.05 ## P<0.01vs cells treated with TGF-β alone.*

4.2.7 ASSOCIATIONS OF TSC22D1 EXPRESSION WITH CLINICAL-PATHOLOGIC FACTORS

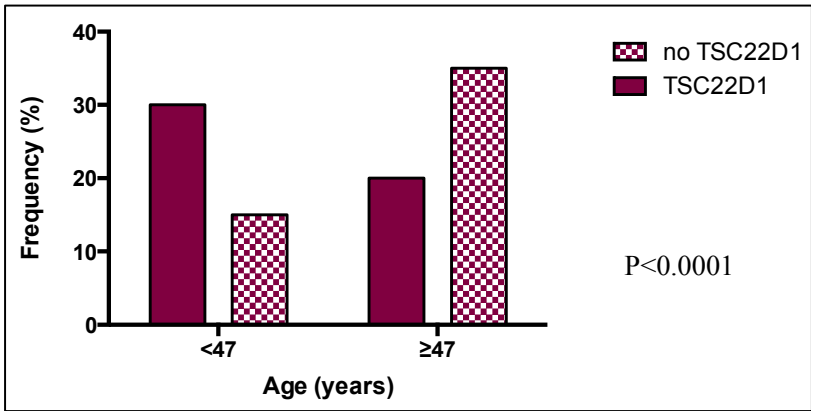
In this study, after assessing TSC22D1 protein levels in 20 BC tissues, we evaluated the association between its expression with clinical and pathological characteristics of the patients. Therefore, TSC22D1 expression was associated with sex, age, Ki-67, tumor size, nodal status and PFS. We found no association between TSC22D1 protein levels with sex (Fig. 30A) and ki-67 (Fig. 30B). On the contrary, a significant association (P<0.001) was found between TSC22D1 expression and age of the patients; particularly, we observed that, the majority of patients whose BC tissues were positive for TSC22D1 expression, were <47 years, while the majority of patients whose BC tissues were negative for

TSC22D1 expression were ≥ 47 years (Fig. 30C). Furthermore, a significant association was found between TSC22D1 expression levels and tumor size and nodal status ($P<0.0001$, respectively). We found that, all the patients whose BC tissues were positive for TSC22D1 expression were characterised by a higher tumor size (Fig. 30D) and a higher nodal status (Fig. 30E) as compared to those patients whose BC tissues were negative for this protein. Particularly, we observed that some patients with BCs positive for TSC22D1 protein expression showed a T2 primary tumor, and involvement of regional lymph nodes, while all patients with BCs negative for this protein showed a T1 primary tumor and no involvement of regional lymph nodes (N0). Finally, we evaluated whether patients whose BC tissues were positive or negative for TSC22D1 protein expression, showed some differences in terms of PFS. As shown in Fig. 30F, we found that the patients with BCs positive for TSC22D1 expression were characterised by a shorter PFS as compared to those patients with BCs negative for this protein. However, a statistical significance was not reached.

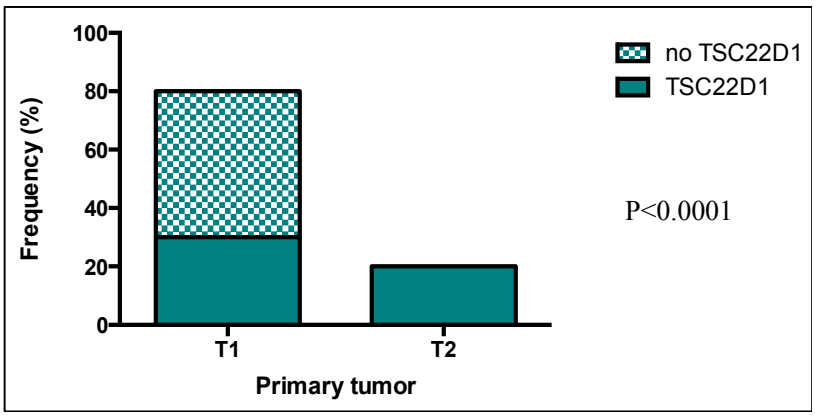




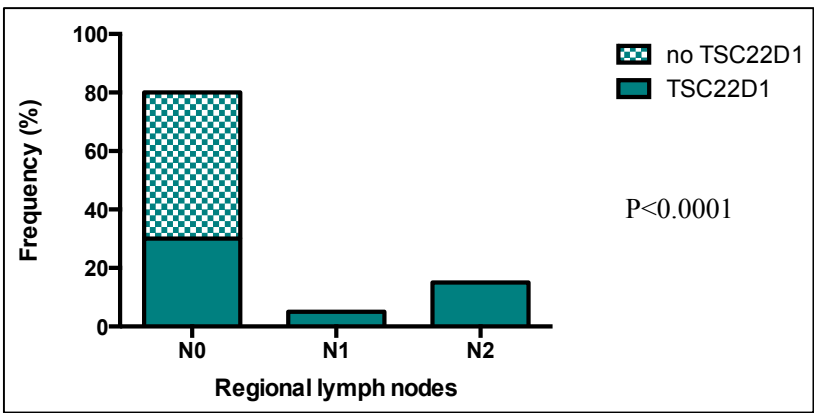
C


40
30
20
10
0
Frequency (%)
<47
≥47
Age (years)
no TSC22D1
TSC22D1
P<0.0001

D


100
80
60
40
20
0
Frequency (%)
T1
T2
Primary tumor
no TSC22D1
TSC22D1
P<0.0001

E


100
80
60
40
20
0
Frequency (%)
N0
N1
N2
Regional lymph nodes
no TSC22D1
TSC22D1
P<0.0001

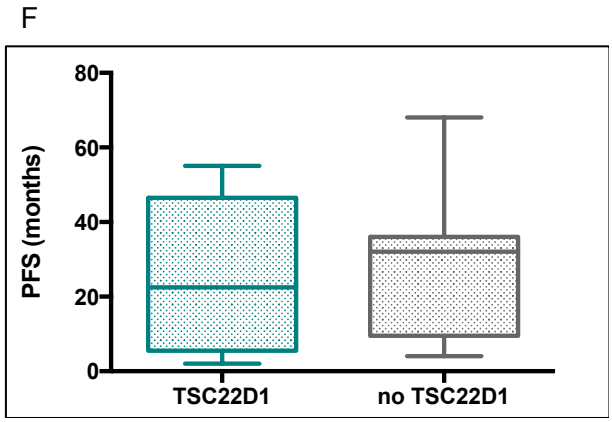


Figure 30: *Association between TSC22D1 protein expression and sex (A), Ki-67 (B) age (C), tumor size (D) nodal status (E), and PFS (F).*

4.2.8 ASSOCIATION OF TSC22D1 EXPRESSION WITH EVEROLIMUS RESPONSIVENESS IN VITRO

In order to understand whether TSC22D1 could be considered a potential marker of resistance to Everolimus, we evaluated the association between TSC22D1 protein levels in frozen BC tissues and the response to Everolimus in BC primary cultures, which were previously defined as responders and non-responders on the basis of at least 20% cell viability reduction (data not shown). Fig. 31 shows that the majority of primary cultures positive for TSC22D1 expression at tissue levels were non-responders to Everolimus in vitro, as well as the majority of primary cultures negative for TSC22D1 expression. Therefore, we found that TSC22D1 protein levels did not associate with responsiveness to Everolimus in vitro.

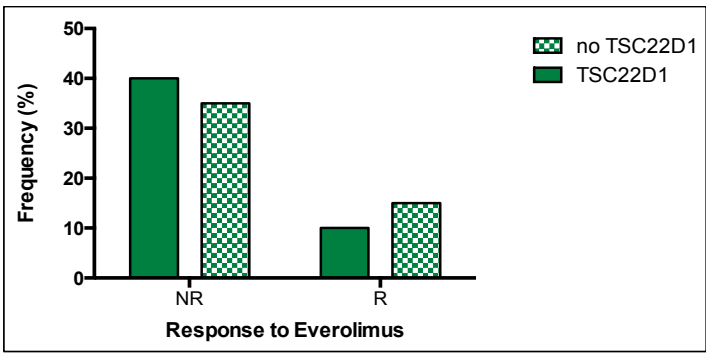


Figure 31: *Association between TSC22D1 protein expression and response to Everolimus in vitro.*

5. DISCUSSION AND CONCLUSIONS

Neuroendocrine tumors (NETs) are a group of neoplasms arising from neuroendocrine cells spread mainly in the respiratory and gastroenteropancreatic epithelium, with various clinical presentations and growth rates. Although these neoplasms have been often reported to be rare, their incidence and prevalence have significantly increased over the past 30 years ([34]). NETs are characterised by the ability to secrete and release bioactive peptides and neurotransmitters into the systemic circulation, which can cause specific hormonal syndromes, depending of the site of origin ([33]). Therefore, when NETs are associated with specific symptoms related to the hormonal secretion, are termed as functioning tumors; in contrast, they are termed as non-functioning tumors when they are not associated with a distinct secretory syndrome ([36]). The tumor heterogeneity, the clinical presentation at advanced stages and the diversity of symptoms contribute to various treatment options ([33]). Radical resection of the primary tumor is currently the only curative option in NET patients, however, as often the diagnosis occurs late, due to the absence of specific symptomatology during the early stage of tumor development, patients present with metastatic disease at the time of diagnosis; in these settings radical surgery is generally not possible ([279]). Chemotherapy and radiotherapy have shown limited beneficial effects in tumor shrinkage or symptom control ([280]), and therefore medical therapy is required. Somatostatin analogs (SSA) remain the most effective pharmaceutical option in functioning NETs, because they reduce hormone-related symptoms ([281]). Although in the last years efforts were made in creating clinical-pathological classification to have an appropriate prognostic and therapeutic tool, currently the molecular biology of NETs is unclear and a univocal therapeutic strategy is still lacking. However, during the last two decades, several targeted antitumor agents including anti-VEGF monoclonal antibodies, tyrosine kinase inhibitors and mTOR inhibitors, which have demonstrated efficacy in several in vitro and in vivo studies, have been developed. The PI3K/AKT/mTOR pathway deregulation is one of the most common mechanisms involved in the pathogenesis of familiar and sporadic NETs, and it is constitutively activated in different NETs ([282]). Phase III trials recently reported the efficacy of Everolimus in patients with advanced P-NETs (RADIANT-3), gastrointestinal NETs and pulmonary NETs (RADIANT-2 and RADIANT-4) ([283]). Although Everolimus produced a significant prolongation of PFS in a number of patients with P-NET and lung carcinoids, it was shown its variable efficacy due to the development of primary or acquired resistance to the treatment ([284]). Therefore, in

the first part of this study our aim was to identify new putative predictive biomarkers in order to understand the mechanisms regulating sensitivity/resistance to target therapies in P-NETs.

On the other hand, in the second part of this study we focused our attention on a gene called TSC22D1; by analysing the differential gene expression profile of typical (TC) and atypical (AC) bronchial carcinoid tissue specimens, we previously noted the down-regulation of this gene in AC samples. It was previously shown that TSC22D1 is down-regulated in prostate cancer as compared to normal prostate ([262]), and a recent study found that the expression of TSC22D1 was significantly decreased in human cervical cancer tissues ([265]), suggesting a role for this protein as tumor suppressor. All these evidences may candidate TSC22D1 as a possible new predictive or prognostic marker in some human cancers. In addition, it was observed that mutations in TSC22D1 gene have been associated with the onset of pulmonary adenomas ([264]). Therefore we aimed at understanding the role of TSC2D1 as prognostic biomarker in bronchial carcinoids in order to identify tumors with more aggressive behaviour, with the goal to develop new therapeutic strategies.

5.1 POSSIBLE PREDICTIVE MARKERS OF RESPONSE TO EVEROLIMUS IN P-NETs

In the first part of this study, we showed that, among 20 human P-NET primary cultures, derived from 16 patients, in 30% (P-NET-R) Everolimus significantly reduced cell viability and induced apoptosis activation in vitro after 48 h, while in 70% (P-NET-NR) Everolimus did not affect these parameters. Even though Everolimus has recently demonstrated its efficacy in prolonging PFS in advanced-P-NET ([169]), and our data did not completely reflect these previous results, it is important to underline that we focused our attention on detection of patients in which Everolimus may be useful in reducing tumor mass, as well as in prolonging PFS, which is clinically influenced by several variables, in order to avoid ineffective treatments in resistant patients. In addition, we found that IGF-1 was capable of influencing the inhibitory effects of Everolimus on cell viability in P-NET-R, but not in P-NET-NR, suggesting an important role for IGF-1 signalling pathway in the mechanism of resistance to Everolimus treatment, which is currently not completely clarified. These results are in agreement with our previous data, in which we demonstrated that IGF-1 blocked the anti-proliferative effects of Everolimus in a group of human

medullary thyroid carcinoma primary cultures ([285]). Furthermore, it has been already reported that GEP-NET display a constitutive expression of IGF-1 and IGF-1R, resulting in autocrine and paracrine growth stimulation, which underlines the important role of IGF-1 in the regulation of NET proliferation ([165,286]). Everolimus specifically targets the mTORC1 complex, without affecting mTORC2 complex. Everolimus has been shown to determine an increase in mTORC2 activity, which directly phosphorylates AKT on Ser473 inducing a rebound AKT activation ([206]). Moreover, mTOR inhibition by Everolimus promotes AKT signalling by reducing p70S6K phosphorylation. The latter is essential to exert a negative feedback on the PI3K/AKT/mTOR pathway activated by IGF-1 ([207]). A correlation between PI3K/AKT/mTOR pathway components expression, clinical outcomes and sensitivity to Everolimus treatment has been suggested in different NETs ([287,217]). We previously observed an evident association between the mTOR pathway profiling and the responsiveness to Everolimus in vitro in bronchial carcinoids; particularly, we found that human BC responders to Everolimus in vitro showed higher levels of AKT, mTOR, p70S6K and ERK 1/2 in their phosphorylated forms, as compared to BC non-responders to the treatment ([213]). On these bases, we tried to understand whether the detection of mTOR signalling pathway components could predict responsiveness to Everolimus in P-NET in order to differentiate those patients who may benefit from this treatment. We found higher levels of p-IGF1R, p-AKT, p-mTOR, and p-4EBP1 in P-NET tissues, whose primary cultures were classified as responders to Everolimus in vitro, as compared to those tissues, whose primary cultures were classified as non-responders, supporting our hypothesis that the resistance to Everolimus in vitro may be related to an inactive AKT/mTOR pathway. On the contrary, in a recent study, Benslama et al. observed that low p-p70S6K protein levels were associated to a better clinical outcome under treatment with Everolimus in NETs ([283]). Our data are clearly not in line with this study, but the latter was conducted considering a heterogeneous group of different NETs, not only P-NETs. Additionally, we also evaluated whether P-NET-R and P-NET-NR may have different clinical characteristics. We found that P-NET-R showed a higher Ki-67 index than P-NET-NR and a trend for a linear correlation between tumor grade and response to Everolimus, suggesting an association between sensitivity to Everolimus and a greater clinical aggressiveness of P-NET.

In order to validate the identified putative predictive markers of response to Everolimus in P-NET, by using a method widely available in other centers, we evaluated p-AKT protein expression by IHC. Subsequently, we correlated the IHC p-AKT positivity with the response to Everolimus in vitro, however, we did not reach statistical significance,

probably due to the low number of samples. The reason why we obtained different results by assessing two different techniques probably could be that, while fresh frozen tissues were used in AlphaScreen assay, IHC was performed using paraffin-embedded tissues. In the first method, before being frozen, fresh tissues were stored several hours in RNA-later solution, which is important to stabilize RNA, but does not modify protein phosphorylation; instead, in the second method, the paraffin-embedded tissues were fixed as large blocks with formalin, that penetrates at 1 mm/h. During this time, the phosphorylation levels of AKT may change ([288]). Finally, comparing Everolimus responsiveness in vitro with p-AKT protein levels assessed by IHC and the response to Everolimus in vivo, we found that, among two patients treated with Everolimus in vivo after surgery for persistence of disease, p-AKT was positive in a patient considered responder in vitro, who showed tumor stabilization after Everolimus treatment in vivo. On the other hand, p-AKT was found negative in a patient non-responder in vitro and who showed tumor progression after 12 months of treatment with Everolimus in vivo. These results show that P-NET primary cultures may represent a good model for testing medical treatment efficacy. Furthermore, our data indicate that IGF-1 pathway is involved in the mechanism of resistance to Everolimus, and candidate p-AKT as a putative marker of sensitivity to Everolimus treatment in P-NET.

5.2 PUTATIVE ROLE OF TSC22D1 AS PROGNOSTIC MARKER IN BRONCHIAL CARCINOIDS

In the second part of this study, we show for the first time differential expression of the TGF-β target gene TSC22D1 in human bronchial carcinoid tissues. We found TSC22D1 protein expression only in 50% of analysed BC samples. This gene encodes for a leucine-zipper transcription factor, originally isolated as a TGF-β inducible gene in mouse osteoblasts ([253]). TSC22D1 has been proposed as tumor suppressor gene since it inhibits growth in brain tumors, prostate cancers and salivary-gland tumors, through mechanisms that are not well understood ([289,290,262]). Furthermore, the tumor progression in several tumor entities has been associated with TSC22D1 down-expression; particularly, this protein was found down-regulated in colon and gastric cancer, cervical cancer, prostate cancer, breast cancer, human brain tumor, hepatocarcinoma, and large granular lymphocyte leukemia ([261]), suggesting that the loss of TSC22D1 expression may have a potential role in malignant transformation. In our study, we observed that TSC22D1

silencing confers a decreased migration capacity in NCI-H727 cells, showing a role of TSC22D1 in influencing the ability of BC cells to migrate. It is well known that increased cellular motility and invasiveness may depend on loss of cell adhesion molecules, including E-cadherin, which is down-regulated in several cancers, such as pituitary adenomas, medullary thyroid carcinoma, and breast cancer ([291,292,293]). Here, we demonstrated that TSC22D1 silencing determined an increase in E-cadherin protein levels, resulting in increased cell-cell interactions, and possibly explaining the decreased migration ability. It has been previously found that TSC22D1 could mediate the growth inhibitory effect of TGF-β in intestinal epithelial cells, probably inducing p21 expression, providing evidences that TSC-22 is involved in the signalling pathway by which TGF-β exerts its anti-proliferative effects. On the basis of these results, we investigated whether TSC22D1 influences the effects of TGF-β on cell viability and apoptosis of bronchial carcinoid cells. We found that TGF-β significantly reduced cell viability and induced apoptosis activation in control cells, while these two processes were not affected by TGF-β in silenced cells, indicating that TSC22D1 plays a pivotal role in affecting the anti-proliferative and pro-apoptotic effects induced by TGF-β. However, it is generally recognized that the role of TGF-β in cancer development is complex, acting both as tumor suppressor in the early carcinogenesis, and as tumor promoter in later stages of cancer progression ([294]). Alterations in TGF-β signalling, such as overexpression of TGF-β and down-regulation of TβR-II, have been reported and associated with poor clinical outcome in prostate cancer ([295]). The exact role of TSC22D1 in TGF-β signalling pathway is currently unknown. Previous studies have reported that TSC22D1 enhanced TGF-β signalling pathway by interaction with Smad 4 in human histiocytic leukemia cell line ([266]). Additionally, it was observed that TSC-22 enhanced TGF-β-induced Smad 2/3 phosphorylation and, then, stabilised and promoted TGF-β pathway, by associating with TβR-I, and decreasing the association of Smad7/Smurfs with the activated receptor in cardiac myofibroblasts ([268]). Therefore, we tried to understand whether TSC22D1 influences the TGF-β effects, by modulating TGF-β signalling pathway components expression in BC cells. Our data show that TSC22D1 silencing did not affect TGF-β and TβR-I protein expression, but decreased TβR-II protein levels. On the other hand we observed that TSC22D1 silencing increased Smad 2, Smad 3, Smad 4, Smad 1 and Smad 6 protein expression. Previous studies have reported an association between the loss of Smad 4 expression and the resistance of Esophageal Squamous Cell Carcinoma to the anti-proliferative effects of TGF-β ([296]). In addition, it was found that a reduction in Smad 2/3 protein levels was associated to a reduced sensitivity to inhibition of growth by TGF-β, but

in the same time, it suppressed metastasis in breast cancer cell lines ([297]). These results are in contrast with our data in which we demonstrated that high levels of Smad 2, Smad 3 and Smad 4 were associated with a resistance to the anti-proliferative effects of TGF-β in silenced cells. However, the control of growth is likely not the only important tumor suppressor end point of this pathway, whose effects are depending on the cellular context. Our results indicate that TSC22D1 is involved in TGF-β signalling pathway regulation and its expression may account for the differences in TGF-β response between control and silenced BC cells. Furthermore, we found that TGF-β treatment did not influence the expression levels of TGF-β pathway components in silenced cells, but it slightly modified Smad 2, Smad 3, Smad 4 and Importin-β protein levels in control cells. We also observed that TSC22D1 silencing determined a decrease in caspase 3 protein expression levels, and that TGF-β treatment led to an increase in caspase 3 protein levels, confirming that TSC22D1 may play a role as pro-apoptotic protein. However, caspase 3 does not reflect the only mechanism by which cells undergo to death. Since a crosstalk between TGF-β and PI3K/AKT/mTOR signalling pathways has been reported ([298]), we also investigated whether TSC22D1 modulates the effects of Everolimus in BC cells. We found that TSC22D1 silencing reduced the anti-proliferative effects of Everolimus; in other words, Everolimus determined a lower reduction of cell viability in sh-NCI-H727 cells, as compared to control cells, suggesting that TSC22D1 may be an important mediator of anti-proliferative effects of Everolimus, as well as of TGF-β. Subsequently, after assessing TSC22D1 protein levels in 20 BC tissues, we evaluated whether TSC22D1 expression associated with clinical and pathological characteristics of BC patients. We found a significant association between TSC22D1 protein expression and age; the majority of patients whose BC tissues were positive for TSC22D1 expression, were <47 years, while the majority of patients whose BC tissues were negative for TSC22D1 expression were ≥ 47 years. This probably may suggest a potential role of TSC22D1 in the early stages of tumor development. Additionally, our data show a significant association between TSC22D1 expression levels with tumor size and nodal status. In other words, we observed that all patients whose BC tissues were positive for TSC22D1 expression were characterised by a greater tumor size and a higher nodal status. According to previous study conducted by Mejer et al., we found that patients with BCs positive for TSC22D1 expression were characterised by a shorter PFS as compared to those patients with BCs negative for this protein; however statistical significance was not reached. On the contrary, we did not find an association between TSC22D1 protein levels and responsiveness to Everolimus in human primary BC cultures.

In summary, all these results show that TSC22D1 silencing causes decreased cell migration, with up-regulation of E-cadherin, and affects the anti-proliferative and pro-apoptotic effects induced by TGF-β, by modifying TGF-β signalling pathway expression. In addition, our results indicate that TSC22D1 seems to associate with a more aggressive behaviour in bronchial carcinoids. Therefore TSC22D1 may be considered as a potential prognostic biomarker in BC patients.

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