Università degli Studi di Ferrara

DOTTORATO DI RICERCA IN
"SCIENZE BIOMEDICHE"

CICLO XXVIII

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INVESTIGATION OF RESISTANCE MECHANISMS TO mTOR INHIBITORS IN BRONCHIAL CARCINOIDS

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1. INTRODUCTION

1.1 NEUROENDOCRINE TUMORS (NETs)

Neuroendocrine tumors (NETs) represent a heterogeneous wide range of different clinicopathological entities and neoplasms, which arise from neuroendocrine cells dispersed throughout the body constituting the so called Diffuse Neuroendocrine System (DNES). The cells of DNES are responsible for the regulation of several physiological events, including metabolic sensing, secretion, absorption, motility, blood flow and proliferation [(1)]. The history of NETs, their discovery and classification, the evolution of their treatment, and the scientists who were responsible for these advancements over the past two centuries are the background upon which we now base our modern understanding of these cancers. Otto Lurbarsch described the first neuroendocrine tumor in 1867 [(2)]; two years later Ransom [(3)] provided the first comprehensive descriptions of the classic symptoms of carcinoid syndrome. The original term of '*karzinoide*' was introduced by Oberndorfer in 1907 [(4)] combined with the primary site in which the tumor occurred in order to distinguish these neoplasms, which he believed were benign, from malignant adenocarcinomata. Subsequently, Gosset and Masson outlined the recognition of carcinoids as endocrine-related tumors in 1914 [(5)]. NETs are usually slow-growing tumors; they can arise from many organs but commonly from Gastro Intestinal (GI) tract and pancreas, lung, thymus and other endocrine organs. NETs may synthesize and secrete peptides and/or amines. These secreted peptides/amines can be used as tumor markers, and they may lead to clinical symptoms. It has become evident that the term *carcinoid* represents a wide spectrum of neoplasms originating from a variety of neuroendocrine cell types. Unfortunately, investigation of the complex nature of endocrine cell function initially was hampered by a lack of experimental techniques applicable to the cell biology of these lesions. The unusual cells of the GI mucosa first attracted the attention of scientist in the second half of the 19th century [(6-7)]. The fact that, these cells can be stained using chromium salt solution, led to them being named enterochromaffin cells. Others preferred to call them, and some still do, Kultschitzky cells, this name deriving from the name of one of the first to discover these cells. Feyrter also described epithelial 'clear cells' (*helle zellen*) in different organ [(8)] and included those with the silver-reducing power (argentaffin cells) described by Masson [(9)]. These cells where thought to exert a local 'paracrine' function by producing amines and peptides and where functionally grouped in a novel system defined as DNES. The histogenetic relationship between the enterochromaffin cells and carcinoid was established once the argentaffin characteristics of some of these tumors were

described [10]. Subsequently interest in DNES cells was revived when the ability of some of them, to undergo amine-precursor uptake and decarboxylation (APUD) was described [11]; in particular was Anthony Pearse (1916-2003) in 1966 who, in 1966, recognized that the endocrine cells of the gut were linked together by a group of common cytochemical characteristics; in particular, the uptake of 5-hydroxytrytophan (5-HTP) and its decarboxylation to 5-HT was analogous in this distinct population of endocrine cells [12]. APUD cells were postulated as being of neuroectodrmal origin, were suggested to be misplaced neurons and were named *neuroendocrine cells* [11]. The DNES was than upgraded to the APUD neuroendocrine system and tumors composed of APUD cells were defined as 'APUDomas'. Nonetheless, the analogies between neurons and endocrine cells stressed by the APUD concept persist in the definition of 'neuroendocrine' markers for those substances identified in both cell lines [13] and for gut DNES cells tumors, commonly defined as 'neuroendocrine'.

1.2 CLASSIFICATION AND HISTOCHEMICAL MARKERS OF NETs

NETs are a heterogeneous group of cancers that have proved to be difficult to assimilate within a globally acceptable classification for more than a century. Different kind of classification systems has been proposed over the past 5 decades, based on embryologic origin [14], morphologic differences [15], or biochemical profile [16]. In 1963, E. D. Williams and M. Sandler proposed the original classification of carcinoid tumors based on their putative embryologic origin (foregut, midgut, or hindgut) [14]. In general, it has been accepted that foregut endocrine cells give rise to carcinoid tumors in the respiratory tract, stomach, first part of the duodenum, and pancreas; midgut carcinoid tumors represent lesions of the bowel from the second part of the duodenum through the ascending colon and appendix; and hindgut carcinoids constitute lesions of the transverse and descending colon and rectum. Carcinoid tumors from different segments of the embryologic gut typically vary widely in terms of the character of their bioactive products, and the differences in secreted agents engender diversity in symptoms and immunohistochemical staining patterns [17]. Subsequently, J. Soga and Y. Yakuwa introduced in 1971 a histological classification based purely on morphological characteristics, describing carcinoid tumors according to their dominant growth pattern: insular, trabecular, glandular, mixed, or undifferentiated [18]. Since 1907 NETs tumors have been considered as a homogeneous group of

malignancies; although well-established in medical terminology, the term carcinoid is no longer adequate to cover the entire morphological and biological spectrum of neoplasms of the DNEs. In the about last three decades efforts were made by the World Health Organization (WHO) to define NETs features; in the first WHO classification for neuroendocrine tumors, published in 1980, the term carcinoid was applied to most of the neuroendocrine tumors. Only the endocrine tumors of the pancreas and thyroid, paragangliomas, small-cell lung carcinomas, and Merkel cell tumors of the skin were differentiated. The carcinoids were divided into enterochromaffin (EC cell), gastrin (G cell), and other unspecified carcinoids [19]. Nevertheless, this first classification often led to misunderstandings between pathologists and clinicians, since the latter employed the term carcinoid to mean a serotonin-producing tumor with carcinoid syndrome while the pathologists applied the term carcinoid to all tumors with neuroendocrine features. In addition, due to the increasing in heterogeneity of these tumors, other difficulties also arose from the diagnosis. For all these reasons the WHO classification, published in 2000 (which was in turn uploaded in 2004 and 2010), introduced the general terms 'neuroendocrine tumor' and 'neuroendocrine carcinoma' [20]. In this classification, a distinction was made between well-differentiated neuroendocrine tumors, which show benign behavior or uncertain malignant potential; well-differentiated neuroendocrine carcinomas, which are characterized by low-grade malignancy and poorly differentiated (usually small cell) neuroendocrine carcinomas 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 [19].

According with the uploading WHO classification published in 2010, neuroendocrine tumors (the term "neuroendocrine neoplasm, NEN, or neuroendocrine tumors, NET" encompasse well and poorly differentiated tumours) have been classified into three main histological categories (Table 2). This classification distinquishes *neuroendocrine tumours grade 1 or NET G1, neuroendocrine tumours grade 2 or NET G2* and *neuroendocrine carcinomas or NEC,* which includes two different subtypes, *of large- or small-cell types;* these poorly differentiated carcinomas are of grade G3. This parallels well with the pulmonary neuroendocrine WHO classification [21]. 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 [(22)].

In this latest WHO classification, the terms "benign" and "malignant" used in the previous classification to describe the well-differentiated tumours, assuming neuroendocrine neoplasms as a category to be potentially malignant, was deleted. On the contrary it has been generally accepted that no histological grading system effectively predicts the behavior of well-differentiated endocrine tumors [(48)]. However, recent studies in well-differentiated NETs of the foregut and midgut have shown the usefulness of a grading system [(23-24)]. Effectively, well-differentiated endocrine tumors with a more solid appearance and distinct proliferative activity, which also lead to difficulties in the differential diagnosis versus poorly differentiated endocrine carcinomas, seem to have a worse prognosis than NETs without these features [(25-26-27)]. For all these reasons, it has been decided to introduce a grading system that could be of help in distinguishing the well-differentiated NETs into G1 and G2 categories, 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 NETs, based on WHO classification for neuroendocrine lung tumors. Adapted from Rindi et al [(48)].

The morphological/biological criteria employed in the NETs classification were tumor size, angioinvasion, proliferative activity and presence of metastases and invasion of adjacent organs. Finally, as a further biological parameter, hormonal activity and association with certain clinical syndromes or diseases were included. Histochemical markers of prognosis include the degree of expression of the proliferation protein Ki-67 and the p53 tumor suppressor protein [(28-29)]. Historically, Ki-67 was incidentally identified as an antibody that bound a nuclear antigen

associated with proliferation in Hodgkin and Reed–Sternberg cells. This measurement of its detection, the Ki-67 index, was used in the assessment of Hodgkin lymphoma [30]. Although discovered more than thirty years ago, the function of Ki-67 is still unclear [31]. 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 [28-32]. Nevertheless, its expression has been noted in cells in all phases of mitosis and despite reservations as to its efficacy, it is used as a surrogate marker of proliferation. It is alternatively named MIB-1 in the literature after the code for the monoclonal antibody that recognizes Ki-67 was produced. The Ki-67 proliferation index refers to the percentage of cells, which are positive by immunohistochemistry for this antigen in a tumor section [28]. This index has been proposed in the WHO classification of GI endocrine tumors (Table 3): (1) well-differentiated endocrine tumor (carcinoid) <2% Ki-67 positive cells, (2) well-differentiated endocrine carcinoma (malignant carcinoid) 2 – 15% Ki-67 positive cells, and (3) poorly differentiated endocrine carcinoma (small cell carcinoma) >15% Ki-67 positive cells [20]. It is of importance to note that in order to perform a proper evaluation of the mitotic count, the pathological specimen must have a minimal size: indeed, 50 HPF (High-power field) represents 10 mm^2. This is not feasible in a biopsy specimen where evaluation of Ki-67 is consequently required. Overall, the prognostic value of this grading was demonstrated for foregut, midgut and hindgut NETs [23-33-34-35-36-37-38-39-40]. The histological grading into G1, G2 and G3 is performed on the basis of the assessment of the proliferation fraction according to the European Neuroendocrine Tumor Society (ENETS) scheme firstly published in 2006. In general, G1 and G2 should refer to well-differenciated 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%), is often associated with fields of necrosis, and shows significantly reduced chromogranin A expression, while maintaining intense staining for synaptophysin [41]. If the grade, concerning the grading system classification, differs for mitosis and Ki-67 evaluation, it is suggested to consider the higher grade.

As for ki-67, considerable research has focused on the tumor suppressor protein p53, as potential histochemical indicators of malignant behavior in neuroendocrine tumors. The p53 gene is one of the most commonly mutated genes in human cancer. Under normal circumstances, the wild-type p53 protein is present in such small quantities that it cannot be detected with use of routine immunohistochemical analysis. In contrast, the mutant p53 protein has a longer half-life and builds up in the cells to immunohistochemically detectable levels. Thus, steady-state p53 protein

levels tend to be higher in cells that have mutant p53 alleles compared with cells without p53 mutations [42]. Overexpression of the tumor suppressor protein p53 is uncommonly identified in GI carcinoids [28]. Several studies of p53 positivity in GI carcinoid tumors have reported detection rates ranging from 0% [43-44] to 19% [43-45-46-47], proving the p53 immunoreactivity can complement histologic grading in predicting the likelihood of biologic aggressiveness.

Although the WHO classification has been an important step towards defining the diverse tumor biology of NETs over the past thirty years, further efforts are necessary to improve the prognostic assessment of each individual NETs. The need for standards in the management of patients with endocrine tumors of the digestive system prompted the European Neuroendocrine Tumor Society (ENETS) to organize a first Consensus Conference in 2006, which was held in Frascati (Rome) and was based on the recently published ENETS guidelines on the diagnosis and treatment of digestive neuroendocrine tumors [48]. Although tumor node metastasis (TNM) staging systems are commonly used in the assessment of tumors, until these guidelines were published, such a system was not available for NETs. Therefore, it followed the first TNM classification which was proposed in 2006 (for NET of the stomach, duodenum and pancreas) and in 2007 (for NET of the ileum, colon/rectum and appendix) by a working group of the ENETS [48-49-50]. 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) TNM classification was published [51], including for the first time digestive neuroendocrine tumours. 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 [52-49]. 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). It is important to document the pathological features, such as invasion and tumor size, to allow the translation of the staging between the classifications [53], Table 4. In detail, as described by Rindi et al. in 2006 [48], the acronym TNM for NET staging proposal, is referred to:

- *T - Primary tumor*: Generally, TX indicate that the primary tumor cannot be assessed while N0 is used when there is no evidence of primary tumor; The size limits indicated for T1 are those defined by the WHO for tumors with “benign behavior” according to site

Pancreas-ENETS		Pancreas-UICC[a]
T1	Tumour confined to pancreas, ≤2 cm	Idem
T2	Tumour confined to pancreas, 2–4 cm	Tumour confined to pancreas, >2 cm
T3	Tumour confined to pancreas and >4 cm or invading duodenum or bile duct	Tumour extends beyond pancreas, without involvement of coeliac axis or superior mesenteric artery
T4	Tumour involves coeliac axis or superior mesenteric artery or adjacent organs (stomach, spleen, colon, adrenal)	Tumour involves coeliac axis or superior mesenteric artery
Stomach-ENETS		Stomach-UICC
Tis	In situ/dysplasia (<0.5 mm)	Idem
T1	Tumour invading mucosa or submucosa, ≤1 cm	Idem
T2	Tumour invading muscularis propria or subserosa or >1 cm	Tumour invading muscularis propria or >1 cm
T3	Tumour penetrating serosa	Tumour invading subserosa
T4	Tumour invading adjacent structures	Tumour penetrating serosa or invading adjacent structures
Small intestine-ENETS		Small intestine-UICC
T1	Tumour invading mucosa or submucosa, ≤1 cm	Idem
T2	Tumour invading muscularis propria or >1 cm	Idem
T3	Jejunum, ileum: tumour invading subserosa Ampulla, duodenum: tumour invading pancreas or retroperitoneum	Idem
T4	Tumour invading serosa or other organs	Idem
Appendix-ENETS		Appendix-TNM[b]
T1	Tumour ≤1 cm; invading submucosa, muscularis propria	T1a: ≤1 cm T1b: >1–2 cm
T2	Tumour ≤2 cm; invading submucosa, muscularis propria, minimally (≤3 mm) subserosa/ mesoappendix	Tumour >2–4 cm or invading the cecum
T3	Tumour > 2 cm or largely (>3 mm) invading subserosa/mesoappendix	Tumour >4 cm or invading the ileum
T4	Tumour invading serosa or other organs	Idem
Colon/rectum-ENETS		Colon/rectum-UICC
T1	Tumour invading mucosa or submucosa, T1a < 1 cm, T1b: ≥1–2 cm	Idem
T2	Tumour invading muscularis propria or >2 cm	Idem
T3	Tumour invading subserosa or mesorectum	Idem
T4	Tumour penetrating serosa or invading adjacent structures	Idem

Table 4: T categories in the UICC and ENETS classifications of digestive neuroendocrine tumors, in the pancreas, stomach, small intestine, appendix and colon/rectum. Adapted from Rindi et al. [48-49], Sobin et al. [51], Bosman et al. [22]. According to UICC, the poorly differentiated NECs are classified as exocrine tumors; [a] According to UICC, all pancreatic NETs (including G1, G2, G3 and well- or poorly differentiated tumors) are classified following the pancreatic exocrine tumor classification. [b] Global cell carcinoids are classified according to the exocrine carcinoma classification.

-specific clinicopathological correlations [20-54-55]. Similarly, for T2 of the stomach and duodenum, the sizes are those indicated for tumors of "uncertain behavior." In the pancreas the size limit given for T2 needs to be validated [54]. Deeply invasive tumors are included under the T3 and T4 definitions, taking into account site-as described specific features;

- *N - Nodes*: N0 indicate the absence of lymph nodes metastases while N1 is referred to the the presence of any single or multiple metastases in regional lymph nodes, according to TNM rules. Although the presence of regional lymph node metastases is, per se, a negative prognostic factor in gastroenteropancreatic NETs [56], the prognostic significance of the number of metastatic nodes is not known.
- *M - Distant metastasis:* M1 indicates the presence of any single or multiple metastases at any distant anatomical site (including non-regional nodes). Because there is evidence that extra-hepatic bone metastases are a particularly ominous sign [57-58], it is recommended to specify the anatomical site of the metastasis according to the TNM classification rules (PUL, pulmonary; HEP, hepatic; OSS, osseous; etc.) [29-51].

1.3 EPIDEMIOLOGY OF NETs

Neuroendocrine Tumors have been usually considered as rare diseases. Due to awareness among physician and diagnostic techniques improvement, like use of topographic testing, high sensitive of immunohistochemical analysis and radiologic diagnostic testing [59], latest epidemiologic studies reported on the US Surveillance Epidemiology and End Results (SEER) database show 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 (1); moreover, the estimated incidence of 5.25 per 100,000 in 2004 is expected to reach to 8 per 100,000 today [60]. NETs are responsible from approximately 0.5% of all cancers [61]. Most of NETs are diagnosed at advanced stages. According to SEER data including 19,669 cases with NETs, 59.9% of NETs arising in gastrointestinal tract were at the localized stage followed by regional (19.9%) and distant stages (15.5%) [15]. Data from Spanish registry showed that gastroenteropancreatic NETs (GEP-NETs) were often diagnosed at advanced stage with 44.2%, followed by localized and regional stages with 36.4% and 14.2%, respectively, and then unknown stage and undetermined cases with 5.3% [62]. However, NETs originating in pancreas tend to be aggressive and about 60% of these tumors are malignant at the time of diagnosis. Even though these diseases may occur at any age, with no significant difference in terms of gender, they commonly arise after the age of 50 years [63]. Distribution of 49,012 neuroendocrine neoplasms, epidemiological data from the SEER 1973–2007 tumor registry database (70), have shown that GEP-NETs constitute the 61% while the resting part, the 39%, represents NON-GEP NETs (see Figure 1 for more details).

Of all GEP-NETs the most common sites are the small intestine (~30.8%), rectum (~26,3%), colon (~17.6%), pancreas (~12.1%), stomach (~8.9%), and appendix (~4,3%) (59-70). Furthermore, the classification of pancreatic endocrine tumors (PETs) in the SEER registry uses the archaic terms 'carcinoid' and 'endocrinomas', which are further subdivided based upon the cell of origin: Insulinomas (from β cells), Gastrinoma (G cells), Glucagonomas (α cells), VIPomas, Somatostatinomas (D cells) and Pancreatic Polypeptidomas (PPomas). In addition, some lesions that do not secrete known bioactive agent, have been called 'Non-Functional' Endocrine tumors [64]. Non-GEP-NETs are principally located in the respiratory system, with about 70% rate incidence, while a minimum part is represented by Urinary (~1%), Breast (~1%), Prostate (~0,6%), and female genitourinary NETs (~4,5%) [18-65-66]. In the SEER registry, ≈1.15% of patients who displayed one NETs were identified as having an additional NET in a second or third organ [67].

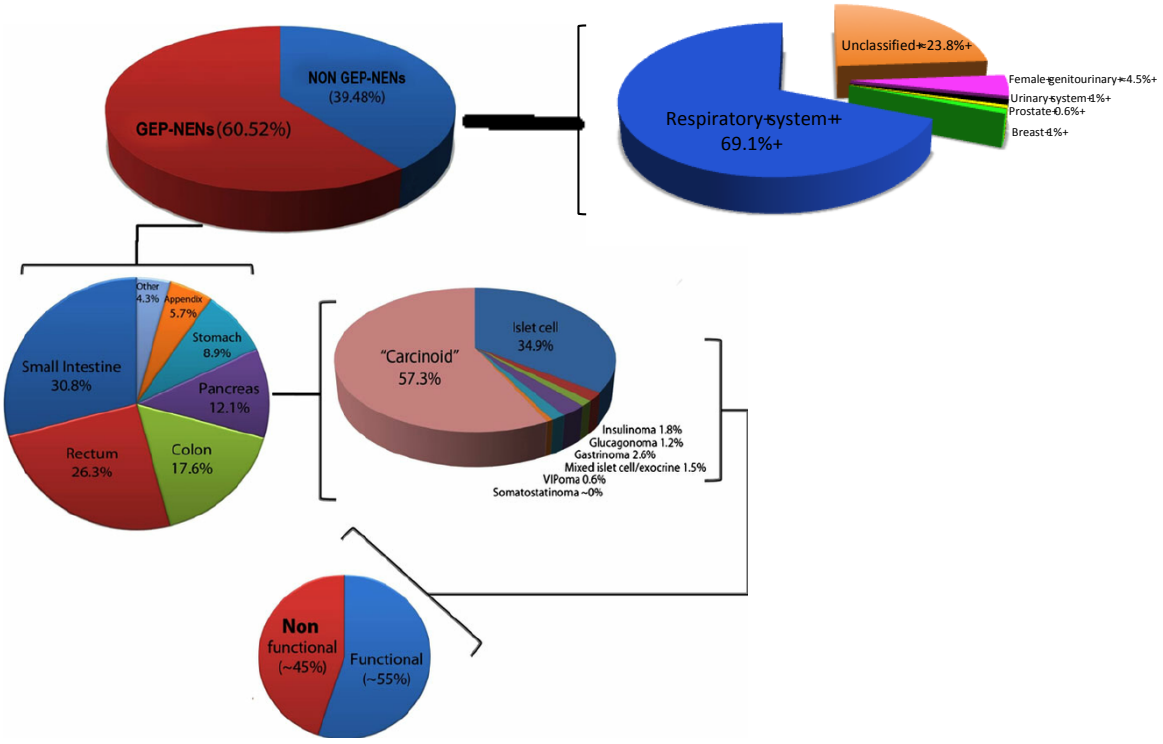


Figure 1: Distribution of 49,012 neuroendocrine neoplasms, epidemiological data from the SEER 1973–2007 tumor registry database. Pie charts reflect the distribution of neuroendocrine tumors by anatomical site and tumor type. (A) Total NEN distribution. (C) Gastroenteropancre-atic neuroendocrine neoplasia (GEP-NEN) distribution. (D) Pancreatic neuroendocrine neoplasia distribution. Non-GEP-NETs are predominantly located in the respiratory system, broncopulmunary NETs ≈70% (B). There is an approximate 1:1 ratio of nonfunctional to functional pancreatic NENs (E)[Adapted from Ref. [14-65-66-70]].

NETs are very heterogeneous tumors. Most of NETs are well or moderately differentiated tumors, with relatively indolent course and slow growth. Thus, overall survival of NETs 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 localizedtumors. The survival has improved in the last two decades. Prognostic factors influencing survival are known as distant metastasis, poorly differentiated tumor, grade, age, number of liver metastasis, extrahepatic metastasis, and the presence of positive surgical margin [60-68-69]. The mean survival across all 49,012 patients with NETs on the SEER database was 58.4% at 5 years, (1973-2002) [70] Table 5.

Location	Within NETs (%)	Within GEP-NETs	Incidence SEER (/100,000)	Incidence (% of primary site)	5-year OS (%)
GEP-NETs	67		5.25	<2	75–82
Gastric		9–20	0.3	1	45–64
Small intestine		39–42	1.1	37–52	62–71
Appendix		6	0.15	30	90
Colon		9–20	0.35	<1	67
Rectum		26	1.1	<1	90
Pancreas		7–12	0.5–1.0	1–2	27–38
Bronchopulmonary	27		0.46	<2	44–87
Other sites[a]	6		0.38		

Table 5: Data SEER database (1973-2002) across 49,012 patients with NETs [70].

NETs originating within the GEP system had a more favourable prognosis than NETs originating in other organs, whit mean 5-year survival of 68.1% and 44.6% respectively [59-70]. Furthermore, while the best survival rates are observed in patients with NETs arising in the rectum and appendix, the prognosis of colonic NETs is worse; the 5-year disease-specific survival rates are about 96% for rectum and 90% for appendix [67-71-103]. Concerning NETs, which arise outside the GEP system, the mean 5-year survival was slightly better in the subgroup of bronchopulmunary NETs at 52,7% [70]. The more aggressive course of NETs outside the GEP system reflect the higher grade, stage at the time of diagnosis and site of the primary tumor, but may also reflect a more malignant behaviour that is s yet not totally clarified at biological level.

1.4 RISK FACTORS AND FAMILIAL SYNDROMES ASSOCIATED WITH NETs

Behavioral features such as smoking [72] and genetic factors [73] have been implicated in the etiology of NETs; however, clear causative factors have not yet been delineated [74]. Although majority of NETs occur sporadically, some may be hereditary and can appear with genetic syndromes (Table 6), such as MEN-1, MEN-2 and von Hippel-Lindau disease [60-75], which is however lower disease-related PETs as compared to MEN-1 [76-77]. Moreover, NETs were less observed in Recklinghausen neurofibromatosis and tuberous sclerosis. Finally, there are other genetic syndromes, characterized by single or multiple endocrine tumors, that were identified and mapped over the past ten years, but the genes related to these diseases, such as Carney complex, non-MEN1 familial isolated hyperparathyroidism (FIHPT), Conn adenoma, and pituitary tumors, remain unidentified [73]:

Name	Neuroendocrine tumor (NET) (frequency)
MEN-1 syndrome (MEN-1) (MIM 131100) (Wermer's syndrome)	Pituitary adenoma (5–65 %)
	Pancreatic NET (80–100 %)
	Thymic NET (mostly ♂), (<10 %)
	Lung NET (20–25 %)
	Gastric, *type 2*, *NET* (ZES related) (5–35 %)
MEN-2a syndrome (MEN-2a) (MIM 171400) (Sipple syndrome)	Medullary thyroid carcinoma
	Pheochromocytoma
MEN-2b syndrome (MEN-2b) (MIM 162300)	Medullary thyroid carcinoma
	Pheochromocytoma
Familiarly medullary thyroid carcinoma (FMTC) (MIM 155240)	Medullary thyroid carcinoma
von Hippel-Lindau (VHL) syndrome (MIM 193300)	*Pancreatic NET* (5–10 %)
	Pheochromocytomas (10–20 %)
Neurofibromatosis I (NF 1, MIM 162200)	*Periampullary NET*
	(*Somatostatinoma*)
	Pheochromocytoma
Tuberous sclerosis (TSC, MIM 191100)	Pituitary adenoma??
	Pancreatic NET?
Carney complex I (CNC1, MIM 160980)	Pituitary adenoma
Carney-Stratakis syndrome (MIM 606864)	Paraganglioma
MEN-4 (MEN-X) syndrome (MIM 610755)	Pituitary adenoma
Familial paraganglioma syndromes (MIM 115310, MIM 168000, MIM 601650, MIM 605373, MIM 614165)	Paraganglioma
	Pheochromocytoma

Table 6: Hereditary tumor syndromes associated with neuroendocrine tumors. MIM: OMIM-online catalog of Merdelian inheritance in man catalog number.

- *MEN-1:* this syndrome results from an inactivating MEN-1 gene mutation localized on chromosome 11q13 [78-79] that encode for Menin, a 67 KDa growth-suppressor protein. The Menin binds JunD, a transcriptional factor belonging to the AP-1 complex, which might be involved in 1–50% of sporadic NETs [73]. MEN-1 syndrome is a multisystem autosomal dominant inherited genetic disorder, characterized by hyperplasia and/or multiple adenomas of the parathyroid glands, single or multiple NET of the pancreas and/or duodenum and stomach, adenomas of the anterior pituitary, NET of the thymus and lung and functioning and nonfunctioning hyperplasia, or adenomas of the adrenal cortex. Less common lesions associated with MEN-1 include cutaneous lesions like angiofibroma, collagenoma, lipoma, and melanoma and peripheral or central nervous system (CNS) tumors such as ependymoma and meningioma [80-81-82].

- *MEN-2*: deregulation of the RET signaling pathways by oncogenic point mutations is responsible for MEN2 syndromes leading to the inherited forms of medullary thyroid carcinoma (MTC). The RET proto-oncogene, a tyrosinekinase receptor, is activated by missense mutations occurring either in the extracellular dimerization domain or intracellular tyrosine kinase catalytic regions. In both cases the receptor is constitutionally activated in the absence of natural ligands [73]. In MEN2A (Sipple's syndrome), MTC is associated to pheochromocytoma (30-50%) and primary hyperparathyroidism (10-20%). In MEN2B (Gorlin's syndrome) the major clinical features are MTC, pheochromocytoma, mucosal neuromas and skeletal abnormalities associated with a marfanoid habitus and ganglioneuromatosis of the gastrointestinal tract. The third variant of MEN2 was defined as familial MTC (FMTC), in which MTC occurs as the sole phenotype in 3 or more patients [83]. The relationship between NETs and MEN2A, as MEN-1, is manly related to genetics mutation that could affect the downstream transduction signaling pathways mediators involved in the pathogenesis.

- *von Hippel-Lindau disease (VHL)*: is a multisystem autosomal dominant inherited genetic disorder that may manifest with retinal angiomas, Central Nervous System (CNS) hemangioblastomas, clear cell renal carcinoma, uni-or bilateral pheochromocytoma(s), pancreatic lesions, endolymphatic sac tumors of the middle ear, and papillary cystadenomas of the epididymis and broad ligament [76-84]. In a recent review of patients affected by VHL, 60% of VHL patients had pancreatic involvement including true cysts, serous cystadenomas, and NET, in ~15% [85-86-87].

- *Neurofibromatosis type 1 (NF-1)*: is a multisystem autosomal dominant inherited genetic disorder characterized by neurofibromas, multiple café au lait spots, axillary and inguinal freckling, iris hematomas, skeletal abnormalities, CNS gliomas, pheochromocytomas, and paragangliomas and occasionally with periampullary somatostatinomas [88]. The NF-1 gene is located on chromosome 17q11.2. It encodes for the protein neurofibromin, which 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 [89].

- *Tuberous sclerosis complex (TSC)*: is a multisystem autosomal dominant inherited genetic disorder, which is characterized by hamartomas in several organs, including the brain, heart, skin, eyes, kidneys, lungs, and liver [90]. TSC is due to by inactivating mutations in either of the two genes TSC; TSC1 is located on chromosome 9q34 and encoding for the protein hamartin, while TSC2 gene, is located on chromosome 16p13.3 and encoding for the gene product tuberin. Mutations of these two genes result in an impaired function of the hamartin-tuberin complex, which in turn lead to the upregulation of the PI3K-AKT-mTOR pathway [91-92].

Data from SEER and Swedish study showed that first-degree relatives of patients with carcinoid tumors are associated with increased risk of also developing a carcinoid tumor [93-94]. Because of the indolent nature of many carcinoids, they often are found incidentally, perhaps in search for other malignancies. This is the reason for a high number of synchronous neoplasms detected at the diagnosis of carcinoids [93-95].

1.5 CIRCULATING BIOMARKERS OF NETs

Gut DNES cells are a broad heterogeneous cells family and include as many as 14 highly specialized epithelial cells [96], Table 7 - Table 8.

The mechanisms, which underlie cells differentiation of the diffuse endocrine-cell system are poorly understood. Transcription factors with a role in neuroendocrine-cell differentiation include protein atonal homolog, neurogenin-3 and neuro-D [97]. Neuroendocrine cells of the DNES arise from pluripotent intestinal stem cells that release a variety of biologically active peptides/amines throughout the paracellular environment and the systemic circulation that may

cause distinct hormonal syndromes. These include serotonin (5-HT), histamine, gastrin, tachykinins and somatostatin among others [98]. The cytoplasm of the neuroendocrine cell is occupied by a large number of secretory granules of varying electron densities, size and shape, which constitute the storage site of secretory products. To date more than 30 gut peptide hormone genes, which express more than 100 bioactive peptides, have been identified. The cells utilize endocrine, paracrine, neuro/auto-crine regulatory mechanisms [99] for amine/peptide-producing; In particular, two regulated secretions' pathway are recognized as controlling the assembly, storage and release of two types of secretory vesicles: the Large Dense Core Vesicles (LDCV), which are the well-known electron dense granule of the endocrine cell; and the Sinaptc-Like Micro Vesicles (SLMV), which are smaller than LDCV and very much similar to the synaptic vesicles of nerve endings [100].

Cell	Main product	Stain	Pa	Stomach		Intestine					
				CF	An	small			Ap	large	
						D	J	I		C	R
P	Unknown	Grimelius, ChgA	f	+	+	+	f	f		f	f
EC	5HT*	Grimelius,PbH, ChgA, Syn	f	+	+	+	+	+	+	+	+
D	Somatostatin	Davenport, PbH, Syn	+	+	+	+	f	f	+	f	+
L	GLI/pYY	PbH, Grimelius, ChgC 1 A, Syn				f	+	+	+	+	+
A	Glucagon	Grimelius, ChgA 1 C, Syn	+	a							
PP	PP	Grimelius, ChgC, A, Syn	+			a					
B	Insulin	Aldehydefuchsin, Syn	+								
X	Unknown	PbH, Grimelius		+							
ECL	Histamine	Grimelius, ChgA, Syn		+							
G	Gastrin	Grimelius, ChgA, Syn			+	+					
CCK	CCK					+	+		f		
S	Secretin	Grimelius, ChgA				+	+				
GIP	GIP					+	+		f		
M	Motilin					+	+		f		
N	Neurotensin	Grimelius, ChgA				f	+		+		

Table 7: Characteristics of the endocrine cells of the human gastroenteropancreatic tract. Pa = Pancreas; CF = corpus fundus; An = antrum; D = duodenum; J = jejunum; I = ileum; Ap = appendix; C = colon; R = rectum;
+ = presence of cells; f = presence of a few cells; a = presence of cells in fetus and newborn;
5-HT = 5-hydroxytryptamine; * = substance P, neurokinins, opioids, guanylin and other peptides; PbH = lead-hematoxylin; EC = enterochromaffin; GIP = gastric inhibitory polypeptide; GLI = glucagon-like immunoreactants (glicentin, glucagon-37, glucagon-29); pYY = PP-like peptide with N-terminal tyrosine amide; PP = pancreatic polypeptide; ECL = enterochromaffin-like cell; CCK = cholecystokinin;
Grimelius, Davenport = silver stains; Chg = immunoreactivity for chromogranin; 1 = heavier staining than; Syn = immunoreactivity for synaptophysin. From Rindi et al. [101].

Tumor type	Main cell type	Pa	Stomach		Intestine						Syndrome
			CF	An	small			Ap	large		
					D	J	I		C	R	
Well-differentiated	B	+									PHH
	A	+									glucagonoma
	PP	+									–
	D	+			+						somatostatinoma
	EC	+	+	+	+	+	+	+	+	+	'carcinoid'
	ECL		+								'atypical carcinoid'
	G	+		+	+	+	+				ZES
	L				+	+	+	+	+	+	–
Poorly differentiated	s/i cells	+	+	+	+	+	+		+	+	

Table 8: Cell types, most frequent sites and possible hyperfunctional syndromes of neuroendocrine tumors of the gastroenteropancreatic tract.
Pa = Pancreas; CF = corpus fundus; An = antrum; D = duodenum; J = jejunum; I = ileum; Ap = appendix; C = colon; R = rectum; + = presence of tumor; EC = enterochromaffin cell; ECL = enterochromaffin-like cell; PHH = persistent hyperinsulinemic hypoglycemia; ZES = Zollinger-Ellison syndrome; – = not defined; s/i = small-intermediate cells. See table 1 for main hormone product of different cell types. From Rindi et al. [101].

Upon specific stimulation, granules are translocate to the cell membrane and their content is released by exocytosis mediated by G-protein-coupled receptors, ion-gated receptors, and receptors with tyrosine-kinase activity. 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 amma-aminobutyric acid (via gamma-aminobutyric acid A receptors) inhibit secretion [102]. The apical part of the neuroendocrine cell frequently communicates with the gut through thin cytoplasmic extensions, which act as mechanosensors and chemosensors that project into the glandular lumen. The secretion is a highly controlled mechanism; peptide hormones are usually packaged into LDCV, which bud from the trans-Golgi network, as prohormones and proneuropeptides and processed just before secretion under Chromogranin A (CgA)-dependent regulation. In particular, peptide and pro-hormones are synthesised in the rough endoplasmic reticulum (RER), together with CgA and other granular proteins; Chromogranins may serve as substrates for proteolytic enzymes and thereby modulate this process. The products are then transported to the Golgi apparatus and packaged into secretory granules (large dense-core granules) [1]. Amines might be also stored in small synaptic vesicles. Other granins (eg, chromogranin-B) regulate proteolytic processing of peptide precursors and promote aggregation-mediated sorting into mature secretory granules, enabling granules to mature into regulatable exocytotic carriers. Secretagogue-evoked stimulation induces actin reorganisation through sequential ordering of carrier proteins at the interface between granules and the plasma membrane. This calcium-dependent step is a prerequisite for regulated

exocytosis, and it allows granule membrane trafficking and release of neuroendocrine contents [1]. Neoplastic transformation of neuroendocrine cells origin NETs but it is unclear whether tumors arise from mature cells or from the initial precursors cells. In some cases they preserve the specific secretory potential of neuroendocrine mature cells, while, in other instances, different agent may be produced [103]. The various neuroendocrine cells' types secreting products are tumor specific and, the presence of these secretory products in the serum can be exploited diagnostically as tumor markers for the diagnosis and follow-up of treatment. Some tumor markers may have prognostic implications. Others might be predictive for treatment response [99-104]; they are subdivided into '*general markers'* and '*specific markers*', depending on the cell type involved [1-99]. The assessment of endocrine differentiation in tumors is obtained using several techniques traditionally including silver impregnation methods and electron microscopy. Such techniques are now rarely applied because of the high degree of reproducibility and the relatively low cost of immunohistochemical methods. In pathology, the diagnosis of a neuroendocrine tumor first relies on the positive assessment of general markers of neuroendocrine differentiation by immunohistochemistry [99-105]. The chromogranin family [106 - 107] and pancreatic polypeptide [108], neuron-specific enolase, NSE [109], and alpha Human Corionic Gonadotropin, HGC, constitute general markers, Table 9, for diagnosis and follow-up of patients with NETs.

Biomarkers	Specificity
CgA, CgB, pancreastatin	H
PP, NSE, neurokinin, neurotensin	I
HCG-α, HCG-β	L

Table 9: General biomarkers of NETs;
H = High, I = Intermediate, L = Low.

A briefly overview of some of most representative:

- *Chromogranin A (CgA):* The granins constitute a whole family, Table 9, of glycoproteins of which CgA and CgB are the most clinically interesting. These proteins are 27–100 KDa in size and contain 10% acidic (glutamic or aspartic acid) residues, as well as multiple single and dibasic amino acid residues [110-111]. CgA is an acidic glycoprotein expressed in the secretory granules of most normal and neoplastic neuroendocrine cell types [112], but also located in the neuronal cells in the central and peripheral nerve system [113]. In adrenal chromaffin cells, CgA and CgB are present in about equal amount, but in thyroid cells and entero-chromaffin cells in the stomach contain mostly CgA and very little CgB [110-111].

Chromogranin A (CgA)
Chromogranin B (CgB)
Secretogranin II (CgC)
Secretogranin III (1B1075)
Secretogranin IV (HISL-19)
Secretogranin V (7B2)
Secretogranin VI (NESP55)

Table 10: The Chromogranin family.

Although CgA is gaining acceptance as a serum marker of neuroendocrine tumors, its specificity in differentiating between neuroendocrine and non-neuroendocrine tumors, its sensitivity to detect small tumors, and its clinical value, compared with other neuroendocrine markers, have not clearly been defined [(114)] and its diagnostic usefulness of this marker is still debatable [(115)]. NETs are usually characterized by increased plasma or serum levels of CgA and sometimes also CgB; effectively, elevated circulating CgA levels have been detected in serum or plasma of patients with various NETs including pheochromocytomas, paragangliomas, PNETs, small intestinal NETs, medullary thyroid carcinomas, parathyroid and pituitary adenomas, and also in a proportion of patients with small-cell lung cancer [(116-117-118-119-120)]. It is also noticed that CgA is more frequently elevated in G1/G2 well-differentiated tumors as compared to poorly differentiated tumors, suggesting a loss of CgA expression in poorly differentiated NETs [(121)]. There is no universal standard calibration for serum or plasma CgA assays. The most useful immunoassays for tumor detection have been those that measure the whole CgA molecule. Assays measuring specifically defined parts of the molecule (pancreastatin) usually have lower sensitivity in detecting patients with NETs. Even if CgA is not a very sensitive marker for diagnostic purpose, since its serum concentrations seem to rise relatively late in the evolution of the tumor, due to the high specificity for NETs detection, it represent one of the best general neuroendocrine serum marker available among others, however, it can have useful clinical management of NETs [(115)]. In particular it may ben useful for those subjects which either no marker is available (so-called nonfunctioning NETs) or the marker is inconvenient for daily clinical use like 24-h urinary 5-HIAA excretions and plasma catecholamines [(114)]. Moreover, the main utility of CgA measurement may be in patient monitoring. Therefore, follow-up prospective data are necessary to examine the performance of CgA assessment in evaluating follow-up and treatment efficacy in GEP NET patients.

- *Pancreatic Polypeptide (PP)*: Pancreatic polypeptide, a 36-amino acid linear peptide, is another general tumor marker secreted by pancreatic polypeptide cells, which are located in

the gut mucosa and pancreas [19]. It has been found to be elevated in NETs of the gastrointestinal tract and pancreas, with a sensitivity of about 50–80% [104-131]. A combination of CgA and PP has been useful in patients with non-functional PNETs, with a sensitivity of almost 95%. A specific meal stimulatory test has been particularly useful in patients with MEN-1 and early detects pancreatic tumors [122].

- *Enolase Neuro-Specific (NSE):* NSE is the neuron-specific isomer of the glycolytic enzyme 2-phospho-d-glycerate hydroxylase or enolase. It is expressed in neurons and neuroendocrine cells cytoplasm and may be useful as a circulating marker for NETs. High NSE expression levels are most frequent in patients with small-cell lung cancer but have also been found to be elevated in 30–50% patients with intestinal NETs, medullary thyroid carcinoma, PNETs and pheochromocytomas [121-123]. In patients with poorly differentiated NETs, NSE might be elevated despite normal CgA levels. Moreover, NSE is also roughly correlated with tumor size although specificity is lower than that of CgA [104-120-121]. The combination of CgA and NSE, as diagnostics circulating markers, has a higher sensitivity than either parameter separately [104-120-122].

- *Human Chorionic Gonadotropin, (HCG):* The human chorionic gonadotropin (HCG), a glycoprotein hormone consisting of alpha and beta subunits, can be ectopically produced by neoplasms. Its alpha and beta subunits have been used as markers to screen for a number of different tumors including NETs. In particular, HCG alpha and beta subunits have been found increased in patients with malignant PNETs [124-125].

- *Other general NETs markers*: Added up to well-known general markers, new ones are gaining acceptance as circulating NETs markers like Pancreastatin or Pro-gastrin releasing peptide (Pro-GRP); the latter is a promising tumor marker for small-cell lung cancer [126]. Other markers, like Cytokeratin fragments (CKfr), could be useful in the management of patients with malignancies of epithelial origin [127] since they are sensitive indicators of tumor cell turnover and also associated with angiogenesis factors that may play a role in NETs [128]. However, limited data are so far presented. Finally, studies have recently identified two isoforms of the ATP-dependent vesicular monoamine transporter (VMAT1 and VMAT2) in large/dense-core granules, providing new positive tools for the diagnosis of gastric enterochromaffin-like cell tumors [129-130].

In addition to general markers, there are *biomarkers specific* for the different functioning NETs. The various tumors and secretarial products are summarized in Table 11.

As previously described, carcinoid tumors have traditionally been divided according to the presumed embryological origin of the precursor cell and together with PNETs collectively considered as GEP-NETs.

Site	Biomarkers	Specificity
Thymus	ACTH	I
Lung	ACTH, ADH, serotonin, 5-HIAA	I
	Histamine, GRP, GHRH, VIP, PTHrp	L
Stomach	Histamine, gastrin	I
	Ghrelin	L
Pancreas	Gastrin, insulin, proinsulin, glucagon, somatostatin	H
	C-peptide, neurotensin, VIP, PTHrp, calcitonin	L
Duodenum	Somatostatin, gastrin	H
Ileum	Serotonin, 5-HIAA	H
	NKA, neuropeptide K, SP	I
Colorectum	Peptide YY, somatostatin	I

Table 11: Site-specific NETs markers; H = high, I = Intermediate, L = Low.

Specific markers for various types of NETs include both structural and functional products which give the basis for another classification system, beside the earlier anatomical classification, in which NETs are also divided into functional tumors that produce a clinical hormone-related syndrome, versus non-functional tumors, which are clinically silent [104-131] and might present symptoms related to tumor growth [117-131].

Furthermore, new potential biomarkers have recently been identified for NETs diagnosis and therapeutic tools; among others, a class of natural occurring small non-coding RNA molecules, the so called micro-RNA (miRNAs), have been correlated, in a study conduced by Li et al. [132], with intestinal NETs tumor progression; in particular, down-regulation of microRNA-133a was related to tumor progression [133] less expressed in lymph node and liver metastases as compared to primary tumor and normal enterochromaffin-like cells [132].

1.6 CLINICAL PRESENTATION: CARCINOID SYNDROME

NETs frequently cause endocrine syndromes by hypersecretion of functional hormones [134]. Because of this, these so-called functioning tumors are often discovered earlier than their "non-functioning" counterparts due to symptoms related to the hormone production. These hormones are stored in secretory granules containing chromogranin A. In fact, a common feature for patients with NETs is elevated levels of chromogranin A in plasma, and measurement of this neuroendocrine marker may be used for diagnosis and follow-up of NETs [41-119-135].

The NETs signs and symptoms usually occur due to hormone and peptide and their metabolites secreting from the tumor cells into the bloodstream [136] generating the so-called carcinoid syndrome. Rosenbaum firstly described the carcinoid syndrome in 1953 [137]; it occurs in less than 10% of patients and affects equally both genders. Symptoms (Table 12) including flushing, wheezing, diarrhea, asthma-like symptoms, abdominal pain, diaphoresis, skin lesions, hypotension, and valvular heart disease, are principal features of the carcinoid syndrome.

Symptom	Frequency (%)
Flushing	85
Telangiectasia	25
Cyanosis	18
Pellagra	2–25
Diarrhea	75–80
Abdominal cramp	75
Bronchospasm	20
Valvular lesions	50
Right heart	40
Left heart	13
Asthenia	<5
Neuropsychiatric symptoms	<5
Musculoskeletal	<5

Table 12: Carcinoids symptoms and their frequencies.

Although most of carcinoid tumors are asymptomatic and silent tumor, they become symptomatic disease when carcinoid tumors metastasized to the liver. In this stage, serotonin and other vasoactive substances secreted by the tumor cells (Table 13*A, B*) escape hepatic metabolization and reach to the systemic circulation. Thus, they are called as carcinoid syndrome.

A

Amines	Vasoactive intestinal peptide (VIP)
Serotonin	Substance P
5-Hydroxytryptophan	Peptide YY
Norepinephrine	Neurokinin A and B
Dopamine	Motilin
Histamine	Kallikrein
Polypeptides	Growth hormone
Gastrin	Neuropeptide K
Corticotropin (ACTH)	Glucagon
Bradykinin	Beta-endorphin
Pancreatic polypeptide (PP)	Neurotensin
Somatostatin	Chromogranin A

B

Location	Secretory products	Carcinoid syndrome
Foregut	5-Hydroxytryptophan histamine, multiple polypeptides	Rare
Midgut	Serotonin prostaglandins, polypeptides	Often
Hindgut	Variable	Rare

Table 13: (A) Substrate causing carcinoid tumors. (B) Primary tumor location, peptides secreted from the tumor cells and frequency of carcinoid syndrome.

The levels of substances released are evaluated by laboratory tests including blood and urine analyses.

2. NEUROENDOCRINE TUMORS OF THE LUNG

2.1 NEUROENDOCRINE SYSTEM OF THE LUNG

Bronchopulmonary neuroendocrine tumors (BP-NETs) represent ~27% of all NETs included in the 1973-2007 SEER registry [70] and approximately ~20% lung cancers and comprise a mixed group of neoplasms with varying degrees of malignancy, arising from neuroendocrine cells of the bronchopulmonary (BP) epithelium [138]. Nowadays, NETs of the lung are thought to arise from the Kulchitzky cells [139], which located in the basal part of the epithelium and deeper layers of the bronchial tree, result in tumors that may grow between the cartilaginous plates as well as endobronchially [135]. Originally recognized in the gastrointestinal tract by their ability to reduce silver salts and later by the presence of secretory/neurosecretory granules seen by electron microscopy [140], neuroepithelial origin of these cells, was firstly supported by the findings of Frölich [141] and subsequently by Lauweryns [142]; Kulchitzky cells have been related by a common neural crest origin to DNES cells [143] of the APUD secretory system [11-144]. The epithelium of intrapulmonary airways in many species harbors diffusely spread innervated groups of neuroendocrine cells, called neuroepithelial bodies, NEBs [139]. The pulmonary neuroendocrine cell system, PNEC, is phylogenetically ancient suggesting a fundamental and relevant function [145]. Although most PNEC exist as solitary cells, some are aggregated in innervated PNEC clusters referred to NEBs [146]. PNECs and NEBs harbor typical endocrine-like dense-cored vesicles that store ATP, serotonin (5-HT), and several neuropeptides, such as gastrinreleasing peptide (bombesin), calcitonin gene-related peptide (CGRP), calcitonin, enkephalin, somatostatin, cholecystokinin, and substance P (SP), [147-148-149-150-151]. During lung development, PNECs are the first cell type to form and differentiate within the primitive epithelium, increasing in number as birth approaches and reaching a peak during the neonatal period; Thereafter, they persist throughout life as a viable population [117-151]. In the healthy adult, PNEC are sparsely distributed, the secretory granule-containing cells are typically tall and pyramidal in shape, extending from the basal lamina of the epithelium and possess apical microvilli projecting into the airway lumen. These microvilli function as the sensory part of the cell and upon stimulation, respond by degranulation and exocytosis of amines and neuropeptides, which exert a local paracrine and neurocrine effect on neighboring cells and activate both extrinsic and intrinsic neurons [117-152], Figure 2.

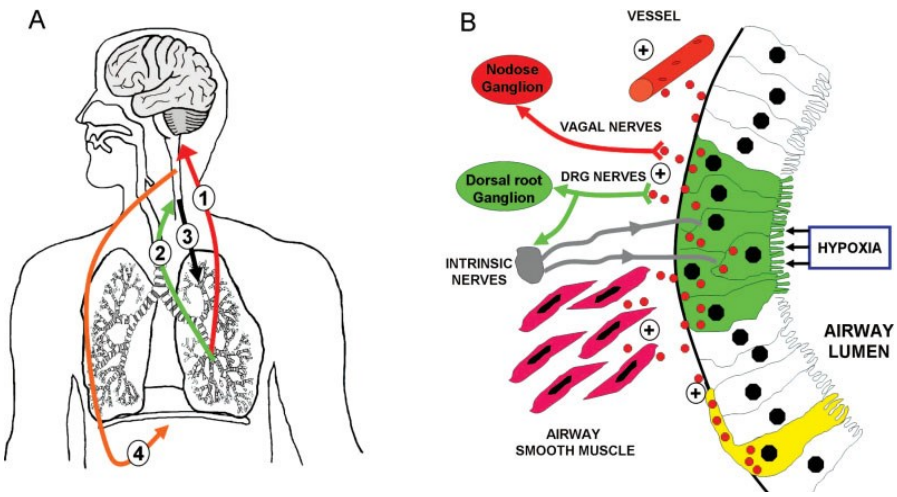


Figure 2: Schematic representation of the role for neuroepithelial bodies (NEBs) as airway sensors. (A) NEB initiated neural regulation modulates pulmonary homeostatic processes including airway tone, pulmonary circulation, and control of breathing. Pulmonary vagal afferent fibers [136] pass to the brainstem, and dorsal root ganglionic (DRG) afferent fibers [153] communicate with the spinal cord. Reflex response signals are transmitted to the lungs via parasympathetic [154] and sympathetic nerve fibers, as well as to the diaphragm via the phrenic nerve [21]. (B) Magnified schematic diagram demonstrating the mechanism of hypoxia-induced degranulation of NEBs (green). The released dense core vesicles (red) contain signal substances including serotonin, CGRP, bombesin, calcitonin, enkephalin, somatostatin, and cholecystokinin, which activate vagal and DRG afferent neurons as well as adjacent epithelial, vascular, or smooth muscle cells. DRGs in turn activate intrinsic efferent neurons facilitating feedback signaling to the NEBs. A single pulmonary neuroendocrine cell (PNEC) (yellow) with basal extension provides paracrine influence on adjacent mucosal cells. Adapted from [117].

Overall, the precise function of the PNEC system remains unclear, as do the functional relationship between PNECs and NEBs.

2.2 CLASSIFICATION OF LUNG NETs

Arrigoni et al. have firstly introduced the traditional classification system, for lung neuroendocrine tumors, during the last decades of last century [155]; among the years, several classifications have been proposed for bronchial carcinoids [13-155-156]. In 1999 a new WHO classification proposed by Travis et al. [157] has been introduced; this classification was than revised in 2004 and apart from the grading also includes a staging system. It defines four histological types based on conventional neuroendocrine morphological (organoid or trabecular growth pattern, peripheral palisading of the tumor cells around the periphery of tumor nests, and the formation of rosette structures) [117] and immunohistochemical features, with different prognostic and therapeutic implications:

1) *Typical Carcinoid (TC)*: Defined as well differentiated neuroendocrine tumors of low malignant potential, TC contain fewer than 2 mitoses per 2 mm^2 and lack necrosis [21] which are usually considered as fairly benign tumors [158]. TC are characterized by organoid, trabecular, insular, palisading, ribbon, or rosette-like growth patterns separated by a fibrovascular stroma. The tumor cells are small and polyhedral with small, round, or oval nuclei and eosinophilic, finely granular cytoplasm. Mitoses are rare or absent, and the proliferative rate is usually low. Furthermore, at electron microscopy, TC shown abundant membrane-bound secretory granules [159].

2) *Atypical Carcinoid (AC)*: Referred to well-differentiated neuroendocrine tumors of intermediate malignant potential, have 2–10 mitoses per 2 mm^2 and/or necrosis [21]. In opposition to the previous ones, AC have a considerable malignant potential [158]. Nuclear pleomorphism, hyper-chromatism, abnormal nuclear-to-cytoplasmic ratio, prominent nucleoli, and areas of increased cellularity with disorganized architecture characterize AC. Mitoses are more frequent, and necrosis may be found. As compared to TC, the AC has fewer granules, distributed in the cytoplasm [159].

3) *Large Cell Neuroendocrine Carcinoma (LCNEC)*: In the WHO classification, LCNECs are considered NETs with >10 mitoses/2 mm^2 and cytological features of a large-cell carcinoma [117]. These neoplasms are usually peripheral and seen as large masses on X-ray. The tumor consists of large cells with moderate to abundant finely granular, eosinophilic cytoplasm and frequent nucleoli. The cells are arranged in organoid, palisading, trabecular, or rosette-like patterns. [21]. Large areas of necrosis are frequent. The proliferative rate is high.

4) *Small Cell Lung Carcinoma (SCLC)*: Identified poorly differentiated NETs of high malignant potential [160] and represent the most aggressive form of lung malignancy. Small-cell lung carcinoma consists of small cells, usually less than the diameter of 3 small resting lymphocytes. The cells are round to fusiform and have a high nuclear-to-cytoplasmic ratio and finely granular, hyperchromatic nuclei with inconspicuous or absent nucleoli. Histologic patterns include trabeculae, spindling, nesting, palisading, rosettes, or solid-sheet-like growth, with indistinct cell borders. The mitotic rate is high, ≥11 per 2 mm^2, median 80 per 2 mm^2. Necrosis and crush artifacts are frequent. The proliferative rate is high [21].

TC and AC are categorized together as carcinoids and represent ~1-3% of all lung cancer [117]; LCNEC is considered a subgroup of large-cell carcinomas, and SCLC is an independent category. The SEER data indicate that LCNECs comprise ~0.3% of all lung neuroendocrine tumors, while others have noted a higher incidence: 1% to 3% of all lung cancers [161-165]. On the contrary, SCLC comprise ~9.8% of all lung tumors [161].

2.3 BRONCHIAL CARCINOIDS (BC)

Bronchial Carcinoid (BC) represents a class of tumors 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 [117]. The term *bronchial carcinoid* has undergone a considerable degree of reappraisal and reclassification over the last 30 years [162]. The report by R. Laennec, published posthumously in 1831 [163], of an intrabronchial mass probably represents the first written description of a BP carcinoid. Hamperl first distinguished these neoplasms, morphologically from other bronchial neoplasms in 1937 [164].

TCs represent 80% to 90% of BP-carcinoids [165] and occur more frequently in the fifth and sixth decades of life; Anyway they can occur at any age, and in childhood are the most common lung tumor. Although they are usually referred to benign-like neoplasms, they may also occur with metastatic spread and behave like ACs, displaying a poorer prognosis, and need to be treated aggressively. In the 1973-2003 SEER registry, right-side BP-carcinoids lesions were the most common ~59.0%, while ~10.4% were located in the main bronchi, Figure 3.

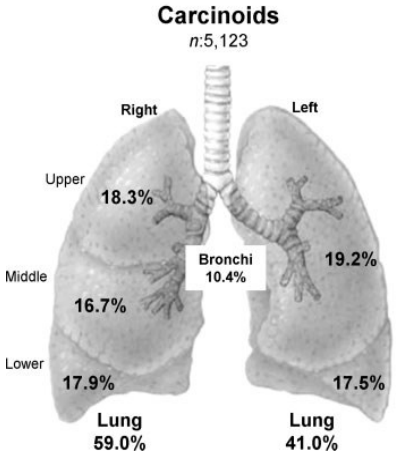


Figure 3: Distribution of BC, registered in the SEER registry [165].As reported from [117].

Davila et al. [166] noted 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 whereas ACs tend to be larger and are more commonly peripherally located [167]. Unlike SCLC and bronchial adenocarcinoma, BCs are not associated with cigarette smoking, ambient radiation, or other known exposure to carcinogens [117].

2.4 EPIDEMIOLOGY OF BC

Bronchial carcinoid (BC) tumors represent a broad clinico-pathologic spectrum neoplasms characterized by a variable morphologic features and biologic behaviors; Their incidence has increased significantly in recent decades (6% per year), due in part to early diagnosis imaging [168]. BC account for approximately 2% of all primary lung tumors and roughly 20% of all well differentiated NETs with an incidence rate ranging from 0.2 to 2 per 100,000 per year [60-169]; In particular the incidence is about 0.7/100,000 in Caucasians and 0.5/100,000 in black people. The disease is slightly more common in women as compared to men [60-67], however, most of them are found accidentally. BC neoplasms occur most frequent in Middle Ages but generally in all ages, even in children and adolescents, in which it represent the most common primary lung neoplasm. The etiology of these tumors is still unknown; in effect, to date there are no clearly defined risk factors for bronchial carcinoids [170] except that patients with MEN-1 syndrome have a higher risk, less than 5% to 10% of cases [171]. Smoking is not a proven risk factor, even if patients with AC are more likely to be smokers as compared with patients with TC [172-173-174]. Recently, genetic risk factors have been associated with BC occurrence. In a gene copy analysis, genome/exome and transcriptome sequencing of BC, some molecular alterations have been identified; the molecular alterations of BC are usually distinct from high-grade NETs, in that they have a lower rate of mutations (approximately 0.4 mutations per mega base), lack *TP53* and *RB1* loss, and contain frequent mutations in chromatin remodeling genes, such as *MEN1, PSIP1,* and *ARID1A* [175]. Although it was previously considered that that BP-NETs represented a continuum, recent information from histologic, immunohistochemical, and molecular studies suggest that the BP-carcinoid group is distinct from the more malignant LCNEC and SCLC groups [21]. The etiology of LCNEC and SCLC is strongly related to tobacco usage, whereas a correlation with BP-carcinoids and tobacco smoking is uncertain.7,8 The 5-year survival rate for BP-carcinoids has decreased drastically (84.7% - 47.3%) over the last 30 years.

3. MEDICAL THERAPY

Remarkable progress has been made over the last several years in knowledge of the molecular biology and treatment of NETs. The field has been transformed from one where patients have limited treatment options to one characterized by an increasing number of clinical trials and approved therapeutic agents. Recent studies have also demonstrated that NETs cannot be longer considered as a single disease entity. Biological differences based on some criteria like the primary site, histologic grade, and ability to secrete hormones and/or other peptides, may influence clinical presentation, prognosis, and response to treatment as a consequence; all these factors must be taken into consideration when formulating treatment plans for individual patients and when designing clinical trials [176].

3.1 SURGERY AND RADIOTHERAPY

The mainstay and only curative treatment of bronchial carcinoids is surgical resection [177]. The principles of surgery include complete removal of the primary tumor, preservation of as much healthy lung parenchyma as possible, and a thorough lymph node dissection, aided by frozen sections, with removal of all affected nodes. For central, localized TC, conservative resection (consisting of sleeve resection, wedge, or segmental resection) is the preferred treatment [178]; controversy exists regarding the optimal procedure for AC and/or lymph node metastases when recognized preoperatively. Since in a study Martini et al. [179] observed the absence of significant correlation between BC survival and lymph node status, they concluded that resection alone was adequate therapy. However, the adequacy of conservative resection and lymph node dissection in AC is still questioned. External radiotherapy is mainly used for palliation of bone or brain metastasis, moreover, since BC are generally resistant to radiation therapy, this modality is reserved in cases with inoperable tumors or as an adjunct when a resection is incomplete [117]. Among others DOTA0-Tyr3octreotate is considered the most effective agent, producing tumor remission in ~50% of BC patients with a median duration of 30 months, in the therapy response in BC, with 90Y-DOTA0-Tyr3octreotide and more than 36 months with 177Lu-DOTA0-Tyr3octreotate [180].

3.2 SOMATOSTATIN ANALOGUES (SSA)

The aims of medical treatment can be defined broadly as amelioration of symptoms and suppression of tumor growth and spread. Somatostatin analogues remain the mainstay of symptomatic treatment for NETs [1]. Most GEP-NETs overexpress all five somatostatin receptors (sst1-2 -3- 4 and 5) [181] while approximately 80% of BC express somatostatin receptors with a predominance of SST2 receptors [182]. Radiolabeled somatostatin analogs (111In-octreotide and 111In-lanreotide) are used in Somatostatin Receptor Scintigrafy (SRS) in order to localize BC [183]; in addition, the development of radioactive analogs for imaging and peptide receptor radiotherapy has improved the management of GEP-NETs [184]. Somatostatin analogs (SSA) are the best therapeutic option for functional NETs because they reduce hormone-related symptoms and may also have antitumor effects [185]; although their effects on tumour growth are limited (less than 5% of patients have objective radiological tumor regression) about 50% of patients have stabilization of tumor size [1]. Encouraging results have been obtained with somatostatin long-acting formulations that lead to stabilize tumor growth over long periods [186]. 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 [187-188].

3.3 CHEMOTHERAPY

Because of the heterogeneity and varying degree of aggressiveness, there is no standard approach to medical or chemotherapeutic management; in addition data regarding the efficacy of chemotherapy in BC are lacking because this tumor type has not been studied independent of other NETs and has been omitted occasionally from such trials [170]. However, various chemotherapeutic agents have been used, including doxorubicin, 5-fluorouracil, dacarbazine, cisplatin, carboplatin, etoposide, streptozocin, and interferon-alpha [189-190], yielding minimal (from ~20% to ~30%) mostly short-lasting results, (from ~20% to ~30%), for suppression of tumor growth and spread [191]. Combination chemotherapies for BC are usually platinum or streptozotocin-based. The initiation of chemotherapy can be controversial because of the slow growth of the tumors and the fact that only palliative treatment is available. When palliative

treatment is offered, it is extremely important to weigh the expected side effects and quality of life against the expected response to treatment. Overall, due to the low response rates for chemotherapy in BC, combined with serious side effects (nephrotoxicity and cytopenia), the indication to use currently available chemotherapeutic regimens in the treatment of BC patient is limited [117]. In general, chemotherapy should be considered for those patients with more rapidly progressing tumors or those who have progressed on less toxic treatments [192-193]; however, additional prospective, randomized studies are needed because treatment principles generally are extrapolated from the experience with the more common GEP-NETs.

3.4 NEW TARGET THERAPY

A better understanding of the mechanisms driving secretion and tumor growth has led to the development of several targeted antitumor agents in these rare tumors. In addition to oldest peptide receptors identified, SSTR and interferon receptors, which inhibitors, introduced in the mid 1980s, represent an established approach to control secretion and growth of NETs and represent the oldest targeted drugs in the management of NETs as well, several other growth-promoting targets are expressed in NET cells [194-195], Figure 4.

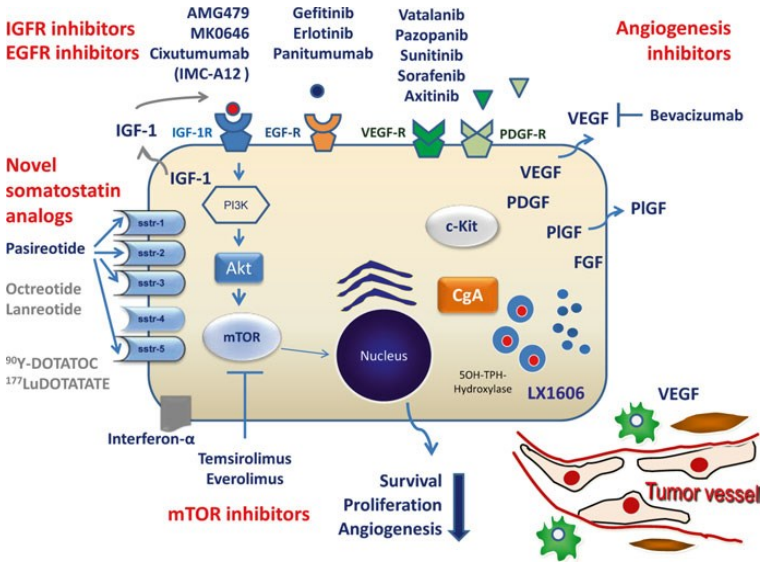


Figure 4: Signaling pathways, drug targets and targeted therapies in NET. As reported from Pavel et al. [196]

These comprise not only growth factor receptors but also their ligands or other molecules, 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-α (TGF-α) and TGF-ß, mammalian target of rapamycin (mTOR) and their cognate receptors [197] among other:

- *Anti-VEGFR therapy*: well-differentiated carcinoid tumors, as well as NETs [198], are highly vascularized and extensively express proangiogenic molecules such as VEGF, hypoxia inducible factor 1a and microvessel density [199]. The antiangiogenic strategies currently used in clinical practice in NETs include monoclonal antibodies against VEGF, such as bevacizumab and tyrosine kinase inhibitors (TKIs) targeting receptors for PDGF and VEGF; the latter comprise Sunitinib, Pazopanib, and Sorafenib that have shown clinical activity in NET [200]. The Pazopanib 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, including patients with bronchial carcinoids [201]. Further antiangiogenic drugs are evaluated in combination with SSA, Everolimus 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 NET, as well as in BC [202], and its binding of EGF or TGF-α induces RAF/MAP-ERK signaling 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 [203]. In vitro and in vivo studies using the EGF receptor inhibitor Erlotinib suggested a potential role and currently phase II studies are ongoing [204]. 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-1 also plays a role in the upstream activation of the mTOR pathway [205-206].

- *Inhibitors of the mTOR Pathway*: The protein kinase mTOR exerts a central control function integrating multiple signaling pathways in response to growth factors and intracellular signaling by nutrients that has recently been involved in NETs pathogenesis

[170]. In NET the PI3K-Akt-mTOR signaling pathway it has been found constitutively activated and this seems to be a crucial event [207] since, its inhibition, play a central role in the new therapeutic strategies for tumor cells growth inhibition. 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 NET [208], while Everolimus, which is well known to have a potent antiploriferative effect in human neuroendocrine tumors [209], have been approved for the treatment of well-differentiated pancreatic NETs based on two multicenter placebo-controlled randomized trials [210 - 211]. The following sections focus on Everolimus and the rule of PI3K/AKT/mTOR signaling pathway in NETs and in particular in BC.

4. EVEROLIMUS AND PI3K/AKT/mTOR PATHWAY IN NETs

4.1 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 [212]; the structurally and functionally conserved mammalian counterpart (mTOR) was subsequently discovered biochemically based on its rapamycin inhibitory properties [213-214]. In every eukaryote genome examined until now, including yeasts, algae, plants, worms, flies and mammals, a TOR gene has been found [215]. Due to the strong homology shared between its C-terminus and the catalytic domain of PI3K, mTOR was included inside the PI3K-related protein kinase family (PIKKs) [215-216-217]. It play a pivotal role, integrating multiple signaling pathways in response to growth factors and intracellular signaling induced by nutrients; in particular, it is involved in the proliferation, survival and other growth-related cellular functions, like the regulation of translation initiation, which is one of the best-known function of mTOR serin/treonin kinase [218]. In addition, another important role has also been identified in the response of cells to hypoxia and energy depletion [219]. mTOR exerts its function by two TOR complexes, Figure 5 [220], mTORC1 (also called raptor) and mTORC2 (also called rictor); whereas mTORC1 complex is strongly inhibited by rapamycin, mTORC2 is not affected by the drug [218]. In yeast, the TORC1 complex regulates transcription, ribosome translation initiation, nutrient uptake and autophagy to the availability of nutrients, whereas TORC2 controls cell polarity and the spatial control of cell growth [221]. In mammalian cells, the TOR complex 1 includes raptor, mLST8, and two negative regulators, PRAS40 and DEPTOR [222-223]; It is activated by the PI3K/AKT pathway and inhibited by the TSC1/TSC2 complex; through the phosphorylation and subsequent activation of S6K on one hand, and the phosphorylation and inactivation of the repressor of mRNA translation 4EBP1 on the other hand, mTORC1 represents one of the major regulator of protein synthesis and ribosomal biogenesis [224]. Since they are the best-characterized downstream mTOR targets, the phosphorylation status of S6K and 4EBP1 are commonly used to evaluate mTORC1 activity *in vivo.* The mTORC2 complex include mTOR, rictor, mLST8, mSin1, Protor, Hsp70 and DEPTOR [225-226]; mTORC2 is activated by growth factors, phosphorylates PKC-α, AKT (on Ser473) and paxillin (focal adhesion-associated adaptor protein), and regulates the activity of the small GTPases Rac and Rho related to cell survival, migration and regulation of the actin cytoskeleton [225-227-228].

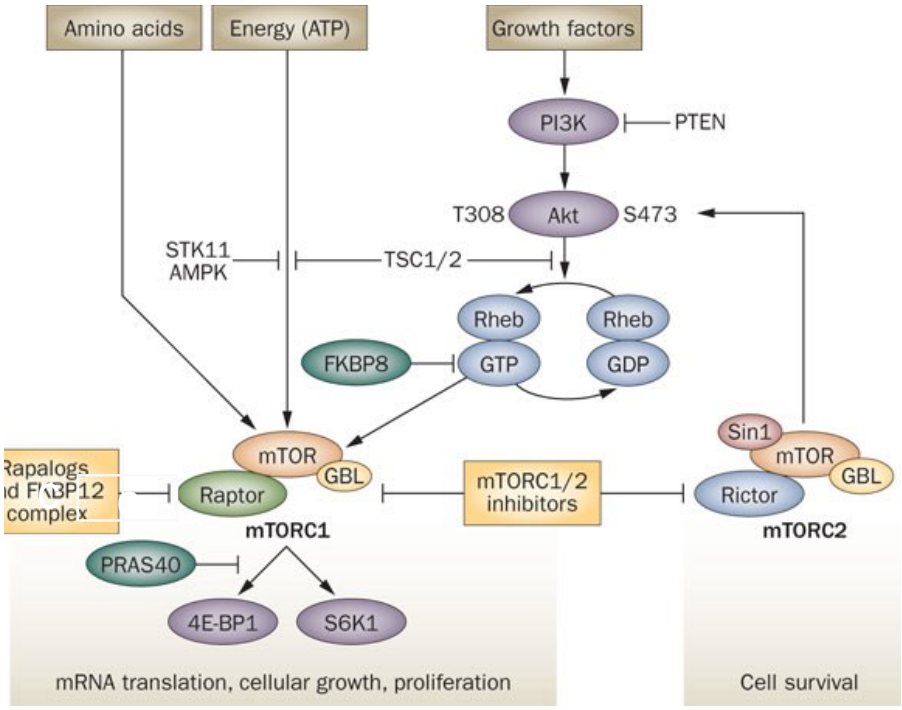
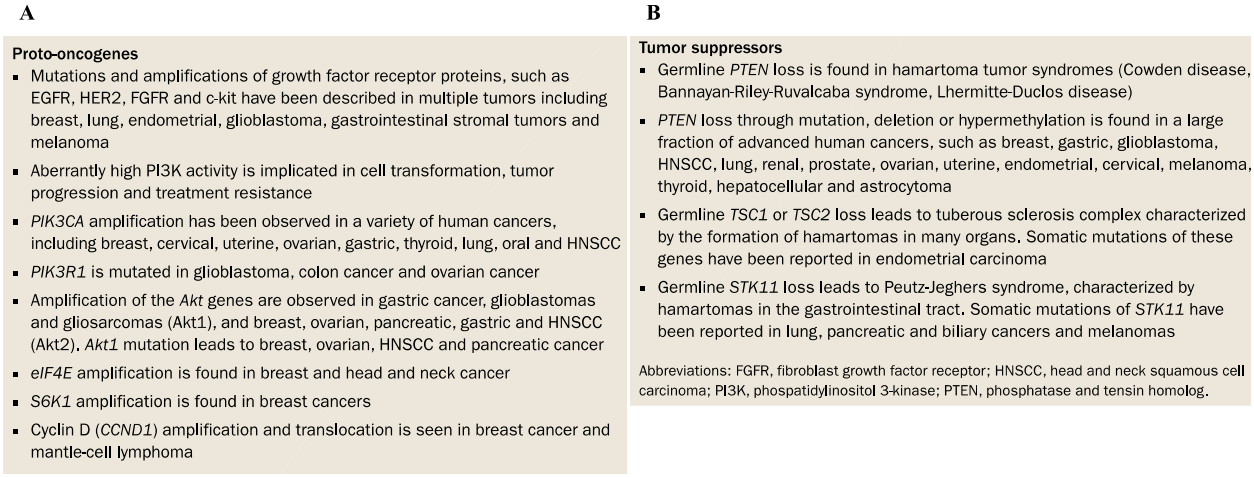


Figure 5: mTOR signaling pathway; ATP, amino acids and signals from the PI3K/Akt pathway modulate mTOR function. Activation of PI3K and Akt inhibits hamartin and tuberin repression of Rheb, which leads to mTORC1 activation and phosphorylation of S6K1 and 4E-BP1. Negative regulators of mTOR include FKBP8, which prevents Rheb from activating mTORC1, and PRAS40, which competes with Raptor for binding to S6K1 and 4E-BP1. Adapted from Dancey J. 2010, Nat Rev. [220].

The mTORC1 signaling cascade is activated by phosphorylated AKT, which needs two phosphorylation simultaneously on Thr308 and Ser473 residues, induced by PDK1 [229] and mTORC2 [230] respectively. Activated mTORC1 phosphorylates downstream effectors, including S6K1 and 4EBP1; S6K1 phosphorylates the 40s' ribosomal protein S6, enhancing the translation of mRNAs with a 5'-terminal oligopolypyrimidine (5'-TOP) [231], while activated 4EBP1 promote its dissociation from eIF4E, relieving the inhibitory effect of 4EBP1 on eIF4E-dependent translation initiation [232], 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 [233]. Differently, in quiescent cells or under low growth factors levels, unphosphorylated 4EBP1 binds to eIF4E, inhibiting the initiation of protein translation.

4.2 THE mTOR PATHWAY AND CANCER

Deregulation of PI3K/AKT/mTOR signaling pathway is one of the most common mechanisms of tumorigenesis [234]. Although specific mutations related to mTOR have not been reported in human cancer, deregulation of upstream pathway effectors can lead to hyperactivation of the mTOR protein, Box 1[220].



A

Proto-oncogenes

- Mutations and amplifications of growth factor receptor proteins, such as EGFR, HER2, FGFR and c-kit have been described in multiple tumors including breast, lung, endometrial, glioblastoma, gastrointestinal stromal tumors and melanoma
- Aberrantly high PI3K activity is implicated in cell transformation, tumor progression and treatment resistance
- *PIK3CA* amplification has been observed in a variety of human cancers, including breast, cervical, uterine, ovarian, gastric, thyroid, lung, oral and HNSCC
- *PIK3R1* is mutated in glioblastoma, colon cancer and ovarian cancer
- Amplification of the *Akt* genes are observed in gastric cancer, glioblastomas and gliosarcomas (Akt1), and breast, ovarian, pancreatic, gastric and HNSCC (Akt2). *Akt1* mutation leads to breast, ovarian, HNSCC and pancreatic cancer
- *eIF4E* amplification is found in breast and head and neck cancer
- *S6K1* amplification is found in breast cancers
- Cyclin D (*CCND1*) amplification and translocation is seen in breast cancer and mantle-cell lymphoma

B

Tumor suppressors

- Germline *PTEN* loss is found in hamartoma tumor syndromes (Cowden disease, Bannayan-Riley-Ruvalcaba syndrome, Lhermitte-Duclos disease)
- *PTEN* loss through mutation, deletion or hypermethylation is found in a large fraction of advanced human cancers, such as breast, gastric, glioblastoma, HNSCC, lung, renal, prostate, ovarian, uterine, endometrial, cervical, melanoma, thyroid, hepatocellular and astrocytoma
- Germline *TSC1* or *TSC2* loss leads to tuberous sclerosis complex characterized by the formation of hamartomas in many organs. Somatic mutations of these genes have been reported in endometrial carcinoma
- Germline *STK11* loss leads to Peutz-Jeghers syndrome, characterized by hamartomas in the gastrointestinal tract. Somatic mutations of *STK11* have been reported in lung, pancreatic and biliary cancers and melanomas

Abbreviations: FGFR, fibroblast growth factor receptor; HNSCC, head and neck squamous cell carcinoma; PI3K, phospatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog.

Box 1. (A) Proto-oncogenes and (B) tumor suppressor genes in the mTOR pathway. As reported from Dancey, 2010 Nature Review [220].

Effectively, proteins regulating mTOR, as well as some of the targets of the mTOR kinase, have been found overexpressed or mutated in human cancer; these mutations includes amplification of the genomic region containing PIK3CA, the gene coding for the p110 alpha subunit of phosphatidylinositol 3'-kinase (PI3K), which has been identified in 40% of cases of ovarian cancer [235] while other mutations, related to PIK3CA as well, have been identified in colon, brain and lung cancers [236]. Moreover, activating mutations may occur in as many as 35% of the cases of breast cancer and is associated with a poor prognosis [237]. The PI3K/AKT/mTOR pathway deregulation has also been involved in the pathogenesis of familial and sporadic NETs [170-238-239] and specifically, mTOR has been identified as a kinase activated in the PI3K signaling pathway of lung NETs [240]. As in other cancer types also in NETs, mutations in the mTOR pathway have been found in approximately 15% of PNETs [239]; for instance loss-of-function in TSC1 and TSC2 tumor suppressor genes, that inhibit mTOR, generally occur in tuberous sclerosis, a hereditary cancer syndrome that has been associated with the development of PNETs [241]; phosphatase and tensin homolog (PTEN), involved in the mTOR activation through the AKT pathway, together with TSC2, are down-regulated in approximately 75% of PNETs, and their low expression is associated with shorter disease-free and overall survival [242]. In addition, in recent studies, mutations of PI3KCA have been also reported in TC and AC [202].

4.3 EVEROLIMUS AND NETs

Although targeted therapy, including mTOR and VEGF inhibitors, being developed for carcinoids arising from the GI tract, treatment for locally advanced or metastatic BCs remains lacking. Traditional cytotoxic chemotherapy offers essentially minimal benefit to these largely under-characterized tumors [243]. In the last decade, Everolimus and Temsirolimus (Figure 6), two rapamycin-derived mTOR inhibitors, have been used for NETs [244] management. The mTOR inhibitors used in clinical practice are derived from Rapamycin, also known as Sirolimus, a macrolide isolated from the bacterium *Streptomyces hygroscopicus,* found in a soil sample taken from Easter Island. Initially studied as an antifungal and antibiotic agent, known for its immunosuppressant activity, it has also showed an anti-cancer proprieties and is usually used as immunosuppressor after organ transplantation [245-246]; as an immunosuppressive drug, rapamycin was approved in 1999 by the FDA for prevention of renal allograft rejection [247]. Subsequent studies reported that rapamycin can also act as a cytostatic agent, slowing or arresting growth of cell lines derived from different tumor types such as rhabdomyosarcoma, glioblastoma, small cell lung cancer, osteosarcoma, pancreatic cancer, breast cancer, prostate cancer, and B-cell lymphoma [215]. In addition to direct anti-tumor effects, rapamycin also inhibits cell proliferation, survival and angiogenesis [248 - 249]. The mTOR-inhibitors can be grouped in two classes: rapamycin and rapamycin analogues that are allosteric inhibitors of mTORC1 and the small molecules that are mTOR kinase inhibitors, TKI [215]. Ever since it has been discovered, several analogues of Rapamycin, modified in order to increase solubility and bioavailability proprieties, have been synthesize; 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) [215]. Temsirolimus, Everolimus and Deforolimus are currently being evaluated in clinical trials for cancer treatment [220]. All rapalogs bind to FKBP12 and preferentially inhibit mTORC1 functions; in particular they act like Rapamycin, forming a complex with the intracellular receptor FKBP12 which in turn binds to mTOR leading to the inhibition of mTORC1 downstream signaling; this inhibition is detectable by the suppression of S6K1 and 4EBP1 phosphorylation [250-251], Figure 6.

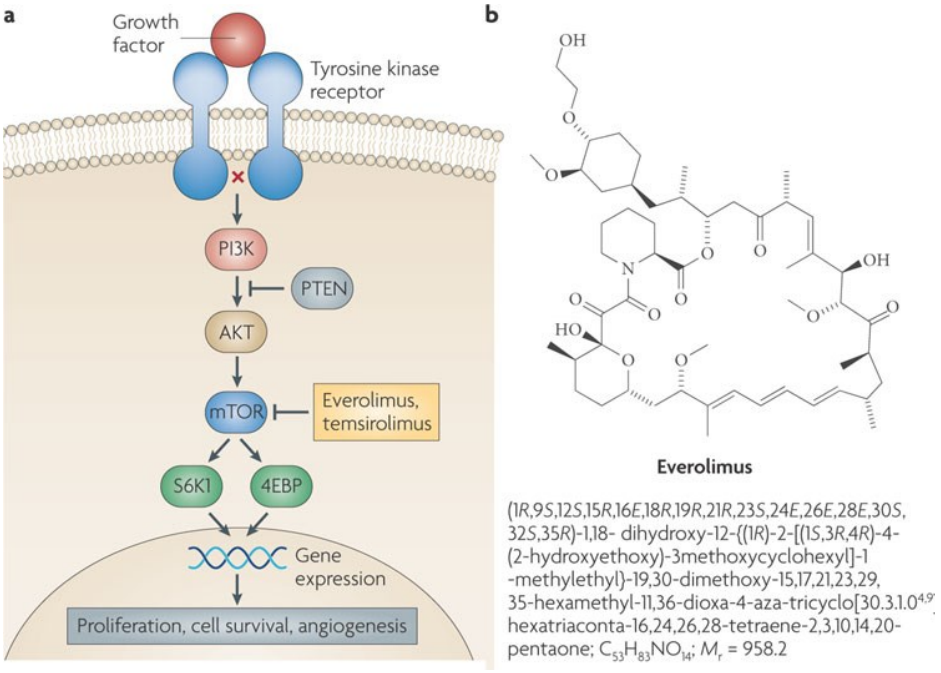


Figure 6: (A) simplified overview of the PI3K–AKT–mTOR pathway, together with points of action of drugs for BCs. (B) Everolimus. Adapted from 2009 Nature Review [252].

In preclinical studies, rapalogs and their analogues have been described to carry out antiproliferative activity in a variety of cancers, not only in NETs, and there are many clinical studies reporting encouraging results in a subset of cancers [253-254] such as Hodgkin lymphoma, non-Hodgkin's lymphoma, breast cancer, endometrial cancer and mantle-cell lymphoma [215]. Noteworthy, Temsirolimus and Everolimus were approved by the FDA for advanced PNETs as well as for the treatment of renal cell carcinoma [255 - 256]; overall, although several new compounds are candidates for molecularly targeted therapy in NETs, only few agents have been evaluated in controlled clinical trials so far. Nonetheless there are results from two phase III clinical trials with the TKI Sunitinib and the mTOR inhibitor Everolimus, for advanced PNETs patients, in which, the combination of both agents, led to an increase of the median progression-free survival (PFS) by 6 months compared to placebo [211-257]; these results had leaded to the Everolimus and Sunitinib approval for advanced well-differentiated PNETs treatment; Everolimus has been also employed in few primary BCs, that have shown a disease stabilization as a consequence [258] but in contrast there are no data on the efficacy of Sunitinib in lung NETs [259]. Subsequently, in a phase III trial RADIANT-2 (RAD001 In Advanced Neuroendocrine Tumors-2), Everolimus was compared with placebo, both in combination with Octreotide-LAR, in a large group of NETs included a subgroup of 44 patients with BCs; Within the lung carcinoid subgroup, the PFS analysis showed a non-significant trend in favor of Everolimus versus placebo [260]; Due to diverging characteristics of the two patient groups, these results must be interpreted

with caution and the next RADIANT-4 study will exactly further evaluate the role of Everolimus in BCs compared to placebo [259]. Sirolimus and Everolimus are oral formulations; Temsirolimus and Ridaforolimus were initially tested with intravenous formulations and oral formulations have subsequently entered in clinical development. The recommended doses for Everolimus and Temsirolimus are 10 mg orally and daily [261] and 25 mg intravenously, weekly [262], respectively. Overall most of rapalogs are well-tolerated in clinical; common toxic effects include skin reactions, stomatitis, thrombocytopenia, diarrhoea, fatigue, hyperlipidaemia and hyperglycaemia while, less common effects include renal insufficiency, peripheral edema, interstitial pneumonitis and infections [263]. The introduction of new therapies in the treatment of NETs can be expected for the future. However, there is still a great need to generate significant clinical data for patients with BCs, which are even rarer than NETs in general, as well as implementation of specific lung NETs registries and cross-linking international research activities are critical for the future of this field. To improve objective response rates and overcome acquired resistance to the drug, multiple combination therapies of Everolimus with somatostatin analogues or angiogenesis inhibitors and dual inhibitors targeting upstream and downstream signaling of the mTOR pathway are currently under investigation. Since other treatments are available (somatostatin analogues, chemotherapy with either Temozolomide or Streptozotocin + 5-fluo, angiogenesis inhibitors), it has still to be defined which patients will benefit most of targeted therapy with Everolimus and in which sequence it should be used.

4.4 RESISTANCE TO mTOR INHIBITORS

Unfortunately, clinical updates indicate that rapamycin shows promise against only a few cancer types. Although Everolimus produced a significant prolongation of PFS in a number of patients with PNETs and lung carcinoids, they do not benefit from the drug owing to early or late progression, probably due to the development of primary or acquired resistance to Everolimus. Rapamycin inhibits mTOR activity but induces upstream signaling, leading to AKT activation, potentially limiting antitumor activity [264], Figure 5. The inhibition of mTORC1 and subsequent phosphorylation of AKT ser473 by mTORC2 was thought to be a potential mechanism of resistance to rapalogs and was one reason for developing mTOR TKI [220]. Two supposed mechanisms of resistance to mTOR inhibitors are AKT and PI3K activation, by means of mTORC2 and IGF1-IGFR signaling, respectively [260]. With ever increasing intensity, resistance to mTOR inhibitors has been related to the rebound AKT activation induced by the negative

feedback mTORC2-mediated; although the study of mTORC2 has just began, the finding that mTORC2 directly phosphorylates AKT adds a new twist in thinking about the role of mTOR in cancer [230-265]. Growth factors stimulate mTORC2 activity and some mTORC2 subunits are phosphorylated, but the responsible kinases remain unknown [225-230-266-267].

The involvement of the mTOR pathway in BCs has been previously suggested by the reported high expression of mTOR and its effector, p70S6K, in a series of metastatic typical BCs [268]. In an additional *in vitro* study, assessed by Moreno et al. [269] on BC-NCI-H727 and PNET-BON1 cell lines, in which cell proliferation was significantly inhibited by rapamycin treatment as a single agent, altering the PI3K/AKT signaling pathway by inhibiting S6K1 and 4E-BP1 phosphorylation with feedback loop AKT activation in both carcinoid cell lines. In other preclinical works, cancer cells with activation of PI3K/AKT signaling, regardless of mechanism of activation, have shown sensitivity to rapamycin [270]. Shah et al. [271] reported that 76% of the NETs demonstrate activation of AKT. Moreover, in line with these evidences, we have previously have demonstrated, Zatelli et al. [209], that mTOR is expressed in advanced BCs, mostly in tissues responding to Everolimus with a reduction in p70S6K activity, indicating on one hand that the mTOR pathway is activated in BCs, on the other hand that Everolimus may activate other transduction pathways, such as AKT itself or ERK pathway, as previously reported [272]; these pathways, in turn, may protect BC cells from the inhibitory effects induced by Everolimus. In addition, the greater mTOR expression in the group of responder BCs has provided the molecular basis for the efficacy of Everolimus, and might explain the resistance of non-responder BCs to the antiproliferative effects of Everolimus, since these tumors express the drug molecular target at lower levels [209]. In keeping with these evidences, in a recent study assessed in our laboratories, Gagliano et al. [273] have confirmed that, approximately 70% of human BC primary cultures respond to treatment with Everolimus in terms of cell viability reduction paralleled with apoptosis activation; furthermore, the '*Sensitive*' BC tissues showed higher total and phosphorylated mTOR serin/treonin kinase as compared to 'resistant' BC tissues. Noteworthy, Everolimus induce an accumulation in the G_0/G_1 phase of the cell cycle only in the '*Sensitive*'' BC cells (NCI-H720). This phenomenon is paralleled by a reduction in the levels of phosphorylated GSK3b, which regulates Cyclin D1 protein levels, which are, in turn, downregulated. However, in the 'resistant' BC cells (NCI-H727), the reduction in both phosphorylated GSK3b and total Cyclin D1 levels does not correspond to a significant cell-cycle phase modification, suggesting that further mechanisms allow 'resistant' BC cells to bypass Cyclin D1 downregulation [273].

5. CELL CYCLE REGULATION AND CKIs REGULATORS

5.1 THE G_0 TO G_1/S CELL CYCLE TRANSITION PHASE

Eukaryotic cells have developed a complex network of cell cycle check-point pathways that protect cells from external stresses and internal errors; in response to any assaults the activation of the network of checkpoint control pathways can lead to diverse cellular responses, such as cell cycle arrest or delay, DNA repair, or elimination of the cell by cell death (apoptosis) if the damage cannot be repaired [274-275]. Depending on the severity and timing, failure of this machinery can lead to embryonic lethality, genetic diseases, and cancer. Most cancer cells exhibit incomplete or malfunctioning checkpoint control pathways that lead to an higher aggressiveness by enhancing potential of cancer cells to survive under suboptimal conditions and increasing their ability to withstand chemotherapeutic intervention. On the whole there are a number of checkpoint, but the three most important ones include: (1) The G1 checkpoint at the G1 transition; (2) The G2 checkpoint at the G2M transition; (3) The spindle checkpoint, at the transition from metaphase to anaphase. The progression through the cell cycle is governed by the periodic activation and inactivation of Cyclin-Dependent Kinase (Cdk) complexes, Ser/Thr protein kinases which activation is regulated by their association partners, called Cyclin [276]. The Cyclin D/Cdk4 and Cyclin D/Cdk6 complexes play pivotal roles in early to mid G_1, whereas Cyclin E/Cdk2 act at the late stage of G_1 [276] and entry in phase S; These Cyclin/Cdks complexes are so defined as positive regulators of G_0 to G_1/S cell-cycle transition. When quiescent cells enter the cell cycle owing to mitogen signals, the expression of the Cyclin Ds is induced and the Cyclin D/Cdk4-6 complexes are formed and then activated by Cdk-activating kinase (CAK) leading to cells progress through the G_1 phase [277]; this allows the complexes to phosphorylate target proteins such as pRB/E2F pathway [277-278] which are the key regulators of the G_1/S transition [279].

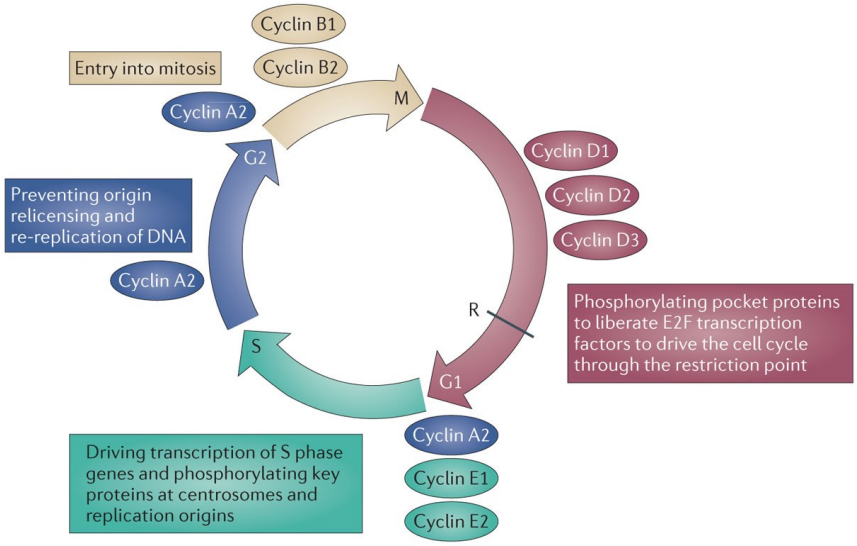


Figure 7: cell cycle control, the different Cdk–Cyclin complexes. D-type Cyclins direct Cdk4-6 to phosphorylate the RB tumor suppressor. Cyclin E/Cdk2 functions at the G_1/S transition to trigger DNA replication and duplication. The Cyclin A/Cdk2 complex collaborates with Cyclin E/Cdk2 to regulate DNA replication both positively and negatively. Finally, Cyclin B/Cdk1 activity reorganizes the cell for mitosis. Adapted from Moore 2013 [280].

The tumor suppressor pRB is the protein required for G_1 cell cycle phase arrest [281] which function as transcriptional repressors in the nucleus by inhibiting the activity of the E2F (early gene 2 factor) transcription factor that regulates the expression of the many genes required for S phase entry and DNA synthesis [282]. In the early G_1 phase, the active ipo-phosphorylated pRB protein is associated with E2F transcription factor; as G_1 progresses, however, the pRB proteins become iper-phosphorylated and so deactivated, primarily by the Cyclin D/Cdk4-6 complexes, but also partly by Cyclin E/Cdk2 [283], inducing E2F releasing and consequent activation; all these findings leads the to S phase transcription genes activation with a cycle progression as a consequence. Most of the G_1/S checkpoint regulators are classified as either tumor suppressors or proto-oncogenes, and their loss-of-function mutations or overexpression appear to play pivotal roles in many human cancer types.

5.2 THE Cdk INHIBITOR $p27^{Kip1}$

Cdk activity is negatively controlled by association with Cdk inhibitors (CKIs); CKIs comprise the INK4 family and the Cip/Kip family which inactivate Cyclin/Cdk complexes and thereby cause growth arrest [278-284]. The Cip/Kip family is composed by broadly acting members such as $p21^{Cip1/WAF1}$, $p27^{Kip1}$, and $p57^{Kip2}$ that inhibit the activities of Cyclin D- E- and A-dependent kinases and induce cell cycle arrest [278].

p27^{Kip1}, encoded by *CDKN1B* gene, is a critical G_1/S cell cycle progression regulator; it was first discovered as an inhibitor of Cyclin E/Cdk2 in cells arrested by transforming growth factor-beta (TGF-β), by contact inhibition, or by lovastatin [285]. Furthermore, it plays a second role in regulating cell cycle progression, facilitating the bond between Cdk4-6 and Cyclin D protein types (but not other Cdks or Cyclins), thereby enhancing this association and promoting the recruitment of the Cyclin D/Cdk complexes to the nucleus [286-287]. p27^{Kip1} is regulated by transcriptional [288], translational [289] and photolytic mechanisms; its proteolysis, Cdk inhibition and assembling functions are regulated by phosphorylation mechanisms [290-291]. To date, five different phosphorylation sites on p27^{Kip1} have been characterized [292], Figure 8;

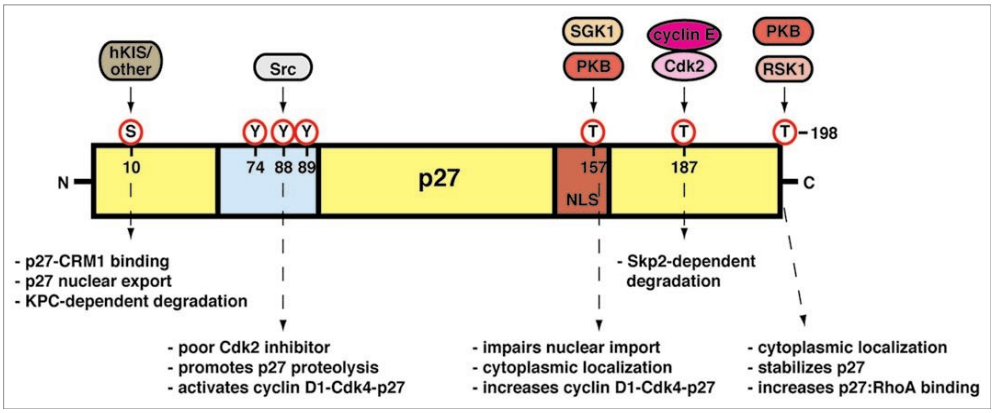


Figure 8: p27^{Kip1} phosphorylation sites. The putative kinases that phosphorylate these specific sites are indicated above each residue. The dashed arrows indicate the role of these phosphorylation events in modulating p27 ^{Kip1} function. Adapted from Larrea et al. 2009 [292].

These phosphorylation sites include: (1) S10, which mediates p27^{Kip1} CMR1 binding and nuclear export to the cytoplasm upon cell cycle re-entry [293-294]; (2) Y74, Y88, Y89, reduce p27^{Kip1} Cyclin E/Cdk2 inhibition and facilitate Cyclin E/Cdk2-mediated T187 phosphorylation and SCFSkp2-dependent p27^{Kip1} proteolysis [291- 295]; (3) T157, whose phosphorylation impairs nuclear import [296] and increases Cyclin D1/Cdk4 assembly [290]; (4) T187, induced by Cdk2 during G_1/S transition [297-298], resulting in p27^{Kip1} dissociation from Cyclin E/Cdk2 complex and its targeting for SCFSkp2-dependent proteolysis [299]; and (5) T198, that regulates p27^{Kip1} stability [300] and increases cell motility [301]. Changes in p27^{Kip1} phosphorylation can lead to loss of p27^{Kip1} protein stability and alterations in its function and/or localization, all of which may contribute to oncogenesis [292]. In effect, the activity of p27^{Kip1} is also controlled by its distribution among different cellular complexes and its cellular localization [302 - 303]. The intracellular localization of p27^{Kip1} can affect the protein's function. Although *wild type* p27^{Kip1} is mainly localized in the nucleus in which can bind and inhibit Cyclin/Cdk complexes, thereby

acting as a cell cycle inhibitor, however, it can be dislocated in the cytoplasm through phosphorylation or protein binding [303], Figure 9 [304]. Once in the cytoplasm $p27^{Kip1}$ may acquire additional functions that are not completely understood even if studies have suggested that it may play a pro-oncogenic role [305], possibly by interacting with Rac and RhoA molecules, which are involved in cell migration [306-307].

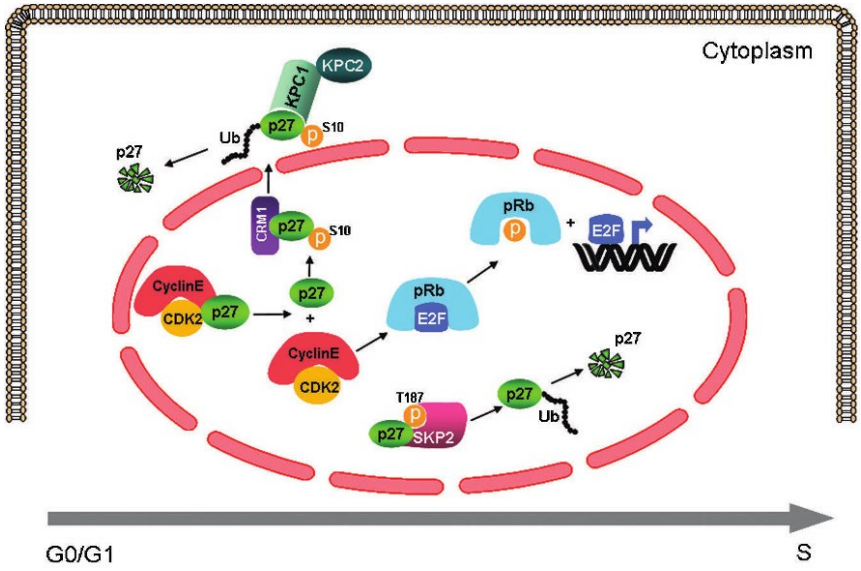


Figure 9: "Graphic representation of the nuclear and cytoplasmic interactions of p27. Upon mitogenic stimulation, p27 is released from Cyclin E/Cdk2 complexes and this dissociation from p27 activates Cdk2, which, in turn, phosphorylates pRb. Phosphorylated pRb releases the transcription factor E2F, which induces the expression of genes required for the G1 to S progression. After the dissociation of p27 from the Cyclin E/Cdk2 complex in early G1 a portion of p27 is phosphorylated on Ser10 and exported into the cytoplasm through the interaction with CRM1 (exportin). Once in the cytoplasm, p27 is ubiquitylated by the KPC1/KPC2 complex and degraded by the proteasome. Upon mitogenic stimulation p27 becomes the substrate of the Cyclin E/Cdk2 complex, which phosphorylates the protein at the Thr187 residue, thereby creating a recognition site for the SKP2 ligase, which promotes ubiquitylation-mediated degradation of p27 by the proteasome in S phase" as reported from Pellegata N, 2012 [304].

Although $p27^{Kip1}$ is degraded efficiently within the nucleus, a study published by Rodier et al. [293] in 2001, have demonstrated that cytoplasmic localization, after phosphorylation at Ser10, is not a prerequisite for its degradation. They found that the fraction of $p27^{Kip1}$ Ser10 is localized exclusively in the cytoplasm of proliferating cells depending on the availability of phosphorylation on Ser10 residue. Moreover, the G_1 $p27^{Kip1}$ Ser10 phosphorylation and the consequent export to the cytoplasm precede the Cdk2 activation, $p27^{Kip1}$ phosphorylation at Thr187 and expression of Skp2 [293]; These findings suggest that $p27^{Kip1}$ export serves to remove this inhibitor to from its nuclear targets, such as Cyclin E/Cdk2, rather than to promote its degradation. Indeed, the $p27^{Kip1}$ Ser10 phosphorylation- and mitogen-dependent translocation to the cytoplasm is required to reduce its nuclear concentration that lead to the activation of free

Cylciln E/Cdk2 and the consequent G_1/S transition [293-294]. In addition, the higher availability of cytoplasmic $p27^{Kip1}$ may also impact on Cyclin D/Cdk4-6 complexes assembling and subsequent activation, promoting G_1/S cell cycle phase as well [293]. Interestingly is that cells with a cytoplasmic $p27^{Kip1}$ are resistant to apoptosis [308].

A number of links between $p27^{Kip1}$ and multiple oncogenic pathways have been established and include the Ras, Myc and AKT/PKB pathways [309]. The phosphorylation at Thr157 residue by AKT is another mechanism, which mediate $p27^{Kip1}$ dislocation to the cytoplasm that has frequently been observed in cancer types such as breast cancer [292-310], and is associated with poorer prognosis. An additional $p27^{Kip1}$ phosphorylation site related to AKT and cytoplasmic protein localization is the Thr198 residue [311].

Due to accelerated proteolysis, impaired translation, sequestration in Cyclin D-Cdks complexes or dislocation to the cytoplasm, in many human cancers loss or reduced $p27^{Kip1}$ protein expression is frequently observed [303] such as breast, colon, lung, prostate, lymphomas and gliomas. Thus is reported to correlate with tumor progression and poor patient survival [312-313].

5.3 THE CDKN1B GENE: POLYMOSPHISMS, MUTATIONS, SOMATIC AND/OR GERM-LINE CHANGES IN HUMAN CANCER

The cyclin-dependent kinase inhibitor 1B gene (*CDKN1B*), codifying for $p27^{Kip1}$ CKI protein, located on chromosome 12, is constituted by three coding region and has four possibly transcripts. Unlike a variety of other well characterized tumor suppressors, $p27^{Kip1}$ gene variations, such as deletions or mutations, are rarely observed in human cancers [314]; although numerous clinical studies have attempted to identify mutations within *CDKN1B* gene in individuals with cancer, such mutations have proved to be extremely rare [314-315]. Instead, several single nucleotide polymorphisms (SNP) have been identified in the human *CDKN1B* gene, including three which are potentially functional: −838C>A (rs36228499), −79C>T (rs34330), and 326T>G (V109G, rs2066827) [316]. Even if epidemiological studies have identified a significant correlation between some of these SNPs and cancer types, such as prostate, breast and oral squamous cell carcinoma, as regard V109G, and papillary thyroid carcinoma as regard rs34330, due to conflicting studies the role of these polymorphism in cancer risk remain controversial [317]. Whilst the down-regulation of $p27^{Kip1}$ protein expression represent the main alterations in human cancers, somatic *CDKN1B* mutations are extremely rare and right now only a handful has been reported in the literature [316]. A nonsense mutation has been identified in an

adult T-cell leukemia/lymphoma (p27W76X) [315], another nonsense mutation was found in a breast cancer sample (p27Q104X) [318] and a missense change in an unclassified myeloproliferative disorder (p27I119T) [319]. More recently, a missense change causing an amino acid substitution (p27P133T) and a 25 nucleotide deletion starting at codons 25 (c.582del25) were identified in sporadic parathyroid adenomas [320].

During the last decade a recessive Multiple Endocrine Neoplasia-like syndrome in the rat (MENX), have been identified [321-322]. The tumor phenotype in MENX rat overlap those of both human MEN-like syndrome type 1 (MEN1) and type 2 (MEN2) that are characterized by tumors involving two or more endocrine glands; animal affected by MENX syndrome develop adrenal pheochromocytoma, multiple extra-adrenal pheochromocytoma, bilateral medullary thyroid cell neoplasia, bilateral parathyroid hyperplasia, and pituitary adenoma; the appearance of neoplastic disease is usually preceded by the development of bilateral juvenile cataracts [321]. Although the spectrum of affected tissues is reminiscent of human forms of MEN, no germ-line mutations were detected in the *Ret* or *Menin* genes that are responsible for the dominantly inherited MEN syndromes in humans; Whereas, as a result of *MenX* locus mapping in rat, Pellegata et al. [322] have identified a *Cdkn1b* germ-line mutation, an 8-bp tandem duplication in *Cdkn1b* exon 2 causing a frameshift which lead to extreme reduction of $p27^{Kip1}$, directly linked to MENX syndrome development. If human mutations in RET is generally detectable in all MEN2 cases, ~ 5-10% of patients affected by a MEN1-like phenotype do not carry MEN1 gene mutations [323-324]. Even if this could be due to the presence of mutations in non-coding or regulatory sequences that are not commonly analyzed, it leaves open the possibility that some other additional mutations may interest susceptibility genes not yet identified. Therefore, following the identification of *Cdkn1b* as the gene predisposing MENX rats to multiple endocrine tumors, individuals with a MEN1-like phenotype but lacking mutations in the MEN1 gene were screened for the presence of CDKN1B germ-line mutations [325]; Thus, a germ-line heterozygous nonsense mutation at codon 76 (c.692G>A, p.W76X) of the human *CDKN1B* gene, in several members of a family with a variant of the MEN1 syndrome, has been firstly identified thereby demonstrating that *CDKN1B* is a tumor susceptibility gene also in humans [326]. An additional germ-line mutation, a frameshift at codon 25 (c.59_77dup, p.K25fs) in a female patient with small-cell neuroendocrine cervical carcinoma, Cushing's disease, and hyperparathyroidism, has been subsequently identified by Georgitsi et al. [327]. More recently, three potentially pathogenic changes in *CDKN1B* (c.-7G>C; c.283C>T, p.P95S; c.595T>C, p.X199QextX*60) were identified in patients with hereditary predisposition to MEN1-like tumors or primary hyperparathyroidism [328]. Therefore, *CDKN1B* mutations, although rare, do occur in patients

with MEN1-related states, the so-called MEN4 syndrome [326]. Overall nine germ-line base substitutions in *CDKN1B* have been so far identified in association with a MEN1-like phenotype. Furthermore, not only patients with a MEN1-like phenotype but also patients with the predisposition to an endocrine disease, such as pituitary adenomas or sporadic presentation of parathyroid adenomas, may occasionally have mutations in *CDKN1B* gene [316].

To conclude, while mutations in *CDKN1B* gene are uncommon in human cancer, the down regulation of the encoded protein $p27^{kip1}$ is often observed in many human tumor types [303] and is usually associate to a poorer prognosis [329-330]. Moreover, reduced $p27^{kip1}$ expression has also been observed in different NETs, including those of the GI tract [331], parathyroid adenoma [332], pheochromocytomas [333], all types of pituitary adenomas [334].

Our understanding of the role of CKI $p27^{kip1}$, involved in NETs predisposition, will improve with the identification and characterization of new germ-line *CDKN1B* mutations. This knowledge may be helpful in devising more focused and effective targeted therapeutic strategies for mutation-positive patients.

6. Cdk-INHINBITOR DINACICLIB (SCH-727965)

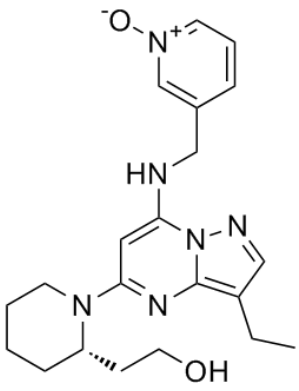
A wide variety of human diseases are characterized by uncontrolled cell proliferation that results from some fault in the copious regulatory pathways that exist in the cell cycle; In particular, alterations of the Cyclin D/CDK4-6/INK4-pRb/E2F cascade, are extremely common in neoplastic cells [(335)]. Overexpression of Cyclins, such as Cyclins D1 and E1, amplification of CDKs, like CDK4/6, inactivation of critical CKIs, loss of Rb expression and loss of binding of CKIs to CDKs, for instance INK4 binding to Cyclin D-dependent CDKs, frequently occur in human malignancies due to chromosomal translocations, genetic mutations or by epigenetic mechanisms [(336-337)]. Cyclin/CDK complexes are, therefore, overactive in most cancers and their pharmacological inhibition thus causes cell cycle arrest [(338)] and induces apoptosis selectively in transformed cells. Therefore, the inhibition of CDKs [(339-340)] has recently emerged as an attractive strategy for the development of novel oncology therapeutics since the inhibition of multiple members of the CDK family has been shown to induce inhibition of proliferation and apoptosis [(338-341)]. The CDK inhibitors (CDK-I) so far known may be broadly categorized into two groups: (a) CDK-I that inhibit CDK1 and CDK2 and (b) CDK-I that inhibit CDK4; in general, it appears to be more difficult to inhibit CDK1 or CDK2 selectively, whereas some moderate success has been achieved in selective inhibition of CDK4:

- *Flavopiridol* (Alvocidib, HMR-1275, NSC 649890, L86-8275, Sanofi-Aventis, Bridgewater, NJ, Paris, France): is a semi-synthetic flavonoid derived from rohitukine, an alkaloid isolated from the leaves and stems of *Amoora rohituka* and *Dysoxylum binectariferum,* plants indigenous to India. It is a pan-CDKI, potently inhibiting at least CDKs 1, 2, 4/6, 7 and 9, as well as a number of other protein kinases [(342-343)], and it also binds to DNA [(344)]. Several phase II trials targeting a variety of indications have been completed. Thus far, the most significant activity has been reported in chronic lymphocytic leukemia (CCL) [(345)]; despite promising single-agent preclinical efficacy against CCL a long-term cytotoxicity was observed; thus has prompted for searching new more efficient combination.
- *R-Roscovitine* (Seliciclib, CYC202, Cyclacel Pharmaceuticals, Berkeley Heights, NJ, Dundee, UK): among the earliest CDK-I described are a class of purines; *R-Roscovitine* is an orally administered, trisubstituted purine derivative of olomoucine that selectively inhibits CDKs 1, 2, 5, 7, 8 and 9 [(336)]; this chemotype has been widely explored by both academic and corporate labs nevertheless, as yet none of these compounds appear to have been advanced to clinical trials for cancer.

Multi CDK-I have been reviewed elsewhere but these agents exhibited insufficient anticancer activity, due in part to the narrow therapeutic window and also to significant toxicity induced [346], probably because these compounds simultaneously block the activity of CDKs required for multiple processes such as transcription, translation, and cell proliferation, and that they may also have inhibitory actions against other classes of protein kinases. These difficulties have focused efforts toward the identification of a second generation of CDK inhibitors with fewer off-target effects and the development of CDK inhibitors that selectively inhibit smaller subsets of CDKs:

- *Palbociclib* (PD0332991, Pfizer, Inc., New York City, NY): is an orally administered, highly specific inhibitor of CDK4 and CDK6, at slightly higher concentrations [347]. It markedly enhances the killing of myeloma cells by dexamethasone and it may also impairs osteoclast progenitor pool expansion and block osteolytic lesion development in Multiple Myeloma (MM) [348]. Finally, it has recently been suggested that *Palbociclib* may both inhibit tumor growth in CDK4/6-dependent tumors and ameliorate the dose-limiting toxicities of chemotherapy in CDK4/6-indepdendent tumors [349].

Dinaciclib (SCH 727965, Merck and Co., Inc., Whitehouse Station, NJ): represents a recent success as regards CDK-I with a broader therapeutic index is a compound developed using a traditional drug-screening program with permutations on a basic scaffold assessed for tumor response in an in vivo model with simultaneous calculation of the therapeutic index, taking Flavopiridol as a benchmark [350] which appears to be both more potent and selective than Flavopiridol, with at least an order of magnitude greater therapeutic index, and is currently in phase III clinical trials. It exerts its effects through the inhibition of various CDKs, in particular it mediate the inhibition of CDKs 1, 2, 5, and 9 with IC50 of 3, 1, 1, and 4 nmol/L, respectively [351], Figure 10



IUPAC: (*S*)-3-(((3-Ethyl-5-(2-(2-hydroxyethyl)piperidin-1-yl)pyrazolo[1,5-a]pyrimidin-7-yl)amino)methyl)pyridine 1-oxide.

FORMULA: $C_{21}H_{28}N_6O_2$

MOLAR MASS: 396.50 g·mol^{-1}

Figure 10: Dinaciclib, molecular structure.

Preclinical studies have shown that it is a potent growth inhibitor in murine xenograft model of human cancer [351-352] and promoter of apoptosis in most cancer cells via suppression of Rb phosphorylation [351]. However, the mechanism underlying its antitumor activity is to be fully elucidated yet; other possible mechanisms besides cell cycle arrest have been suggested [353-354]. Given its higher selectivity and efficacy at low nanomolar concentration, Dinaciclib appears to be promising for further clinical drug development. Preclinical testing, Box 2, suggested greatest efficacy against leukemias [355] while, in patient-derived CLL cells it promotes apoptosis and abrogates microenvironmental cytokine protection [356]. *Dinaciclib* potently inhibited the growth of acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) cell lines *in vitro* [357]. Furthermore, in preclinical studies assessed on breast cancer cell lines and murine xenograft models, has been also observed that it may induce apoptosis and tumor regression [351-358].

Preclinical studies	
Broad spectrum of human solid tumors and hematological malignancies [45]	Inhibition of tumor growth Apoptosis in many human cell lines, even after a single exposure Regression of established solid tumors in mouse models Short exposure to dinaciclib can induce long-lasting pharmacodynamic effects in vitro
Osteosarcoma [47]	Apoptosis of several osteosarcoma cell lines, including those resistant to doxorubicin and dasatinib, at low nanomolar concentration of dinaciclib
Melanoma [68]	Inhibition of tumor growth and tumor regression in human melanoma mouse xenografts Apoptosis in a large panel of melanoma cell lines through a mechanism requiring p53 expression
CLL [69]	Apoptosis of CLL cells independent of high-risk genomic features, downregulating mRNA and protein expression of anti-apoptotic protein MCL-1, which has been shown to be essential for CLL cell survival
Pancreatic cancer [46]	Significantly reduced in vitro cell growth, motility and colony formation Reduced subcutaneous tumor growth in 100% of human pancreatic cancer xenografts Treatment of pancreatic cancer xenografts with dinaciclib and gemcitabine was significantly more effective than either agent alone
Childhood cancers (including solid tumors and leukemia) [70]	Dinaciclib has shown to arrest cell growth in most of the cell lines, but it did not induce objective responses in the solid tumor xenograft panels (the best response observed was stable disease for one osteosarcoma xenograft). In the leukemia panel, there were two objective responses (one complete response)
Clinical trials	
Advanced solid tumors, hematological malignancies [50]	Nonrandomized, Phase I clinical trial with dinaciclib administered weekly as a 2 h infusion. There were no observed complete or partial responses. Recommended Phase II dose was 12 mg/m^2. The primary dose-limiting toxicities were sepsis, hyperuricemia and hypotension
Advanced malignancies [51]	Nonrandomized, Phase I clinical trial with dinaciclib administered in a 21-day schedule. No objective responses observed, but prolonged SD was achieved in some patients. Recommended Phase II dose was 50 mg/m^2, without pharmacocynetic interaction with aprepitant. The primary dose-limiting toxicities were neutropenia and transient liver function alteration
Non small cell lung cancer [71]	Randomized, multicenter Phase II clinical trial on patients previously treated for NSCLC. Patients were randomized to receive intravenous dinaciclib monotherapy (50 mg/m^2) or oral erlotinib (150 mg/m^2). No benefit in time to disease progression has been found in with dinaciclib treatment compared with erlotinib. No objective response rate was observed. Dinaciclib treatment was generally well tolerated
Relapsed/refractory acute lymphoid or myeloid leukemia [64]	Dinaciclib monotherapy (50 mg/m^2) given intravenously every 21 days was compared with gemtuzumab ozogamicin in patients with relapsed/refractory acute lymphoid or myeloid leukemia. Dramatic but transient reduction in circulating blasts was observed, but no remission. Toxicities reported were gastrointestinal, fatigue, transaminitis, tumor lysis syndrome (including 1 patient out of 20 treated with dinaciclib who died from renal failure)
Advanced breast cancer [63]	Randomized, multicenter Phase II trial comparing dinaciclib (50 mg/m^2, intravenously every 21 days) with capecitabine (1250 mg/m^2, orally, twice a day in 21-day cycles). Dinaciclib monotherapy had acceptable safety and tolerability. Grade 3 and 4 treatment-related adverse effects included neutropenia, leukopenia, increase in transaminases and febrile neutropenia. Efficacy was not superior to capecitabine

CLL: Chronic lymphocytic leukemia.

Box 2: Preclinical and Clòinical data on Dinaciclib; as reported from Criscitiello et al. 2014 [358].

Our increasing understanding of the molecular mechanisms underlying cell cycle deregulation in cancer suggests the therapeutic value of targeting CDKs. Although CDK-I finally appear to be poised to have clinical impact, and this has been made possible through the development of more selective and potent ATP-competitive CDK-I, it remains to be investigated yet whether wide spectrum CDK-I could be more effective than the highly selective ones as anticancer therapies, and which CDK should be targeted to develop new effective therapies. This avenue will likely yield new and useful drugs for the treatment of cancer and other proliferative diseases.

7. AIM OF THE STUDY

Bronchial Carcinoids are rare tumors originating from endocrine cells dispersed in the respiratory epithelium that still lack of medical therapy. Traditional cytotoxic chemotherapy offers essentially minimal benefit to these largely under-characterized tumors [243]; to date, the mainstay and only curative treatment of BC is complete surgical resection [117] of the primary tumor with the preservation of as much healthy lung parenchyma as possible. As these neoplasms are often asymptomatic and silent tumors, the diagnosis often occurs accidentally, when a lymphonodal and metastatic invasion is already displayed; in these circumstances patients are inoperable and medical treatments are required. Despite somatostatin analogs represent the best therapeutic option for functional NETs they usually reduce hormone-related symptoms seldom showing an antitumor effects [185]; thus, it became evident the necessity to dispose of new medical treatment which might be effective in controlling the various features of the disease. A better understanding of the mechanisms driving secretion and tumor growth has led to the development of several targeted antitumor agents in these rare tumors; among the others, mTOR inhibitors have been used for NETs [244] management during the last decade. Unfortunately, clinical update shows that a number of patients do not benefit from the drug likely due to the development of primary or acquired resistance to Everolimus [264]. We have previously demonstrated [273] that approximately 70% of human BC primary cultures respond to treatment with Everolimus in terms of cell viability reduction paralleled with apoptosis activation. Furthermore, in the human BC cell line NCI-H720, which is *Sensitive* to mTOR inhibitors treatment, Everolimus induced a downregulation of Cyclin D1 protein levels with a parallel greater cell accumulation in G_0/G_1 cell-cycle phase. On the contrary, in the *Resistant* BC cell line, the NCI-H727 cells, the reduction in total Cyclin D1 protein levels does not correspond to a significant cell-cycle phase modification, suggesting that further mechanisms allow *Resistant* BC cells to bypass Cyclin D1 downregulation and cells continue to proliferate.

The mechanisms underlying this phenomenon have not been fully clarified therefore the aim of our study was to further investigate the mechanisms involved in the resistance to mTOR inhibitors in human bronchial carcinoids, trying to identify new putative medical treatments; for this purpose experiments have been performed on:

1. Two human cell lines, NCI-H720 (Atypical Carcinoid) and NCI-H727 (Typical Carcinoid);
2. Human primary coltures obtained from a group of bronchial carcinoid patients' tumor tissues, collected immediately after surgical resection;
3. Genomic DNA extracted from peripheral lymphocytes of 14 BC patients;
4. Somatic DNA extracted from primary tumor tissue fragments, immediately frozen in liquid nitrogen under ribonuclease-free condition at the time of surgery;
5. Histological tissues samples for IHC.

8. MATHERIAL AND METHODS

8.1 DRUGS AND CHEMICALS:

Everolimus (RAD001) [40-O-(2-hydroxyethyl)-rapamycin] was provided by Novartis Pharma (Basel, Switzerland); the compound was dissolved in dimethyl sulfoxide (DMSO) and stored at -20 °C as 10^{-2} M stock solution (i.e. 9,58 mg/ml). Dilution to the final concentration was made in culture medium immediately before use.
Dinaciclib (SCH727965) (2S)-1-[3-Ethyl-7-[[(1-oxido-3-pyridinyl)methyl]amino]pyrazolo[1,5-a]pyrimidin-5-yl]-2-piperidineethanol was provided by Selleckchem (Canada, USA); the compound was dissolved in dimethyl sulfoxide (DMSO) and stored at -80 °C as 5×10^{-3} M stock solution (i.e. 5 mg/ml). Dilution to the final concentration was made in culture medium immediately before use.
All other reagents, if not otherwise specified were purchased from Sigma (Milano, IT).

8.2 *IN VITRO* IMMORTALIZED HUMAN BC CELL LINES:

The NCI-H727 and NCI-H720 human bronchial carcinoid (BCs) cell lines were purchased form the American Type Culture Collection (Manassas, VA, USA). NCI-H727 cells derive from a Typical Carcinoid of a 65 years old Caucasian female; NCI-H720 cells derive from an Atypical Carcinoid of a no-smoker male donor; both BC cell lines were cultured and maintained in RPMI 1640 medium (Euroclone, Milano, IT) implemented with 10% of Fetal Bovine Serum (FBS) at 37 °C in a humidified atmosphere with 5% of CO_2.

8.3 HUMAN BC TISSUE COLLECTION AND PRIMARY COLTURES

Tissue samples of 22 patients (13 males and 9 females; age = 49.6 ± 3.8 years; median = 47.5; clinical characteristics are shown in Table 14) diagnosed and operated on with BC were collected following the guidelines of the local committee of human research, at the University of Ferrara in collaboration with the Section of General and Thoracic Surgery, Department of Morphology, Experimental Medicine and Surgery, and the and University of Padua, Department

of Medical and Surgical Sciences. Histological and immunohistochemical diagnosis was performed according to the WHO classification [359].

#paz	SEX	AGE	BIRTH	SIDE	Ki67	TNM	Diameter (cm)	Histology
#1	M	52	16/03/1958	DX	25%	pT1aN0Mx	1,3	Atypical Carcinoid
#2	F	42	17/06/1968	SX	2%	T2N0Mx	3,5	Typical Carcinoid
#3	M	69	12/06/1941	DX	<1%	T2aN0Mx	3,5	Typical Carcinoid
#4	M	70	06/07/1940	DX	10%	T1N2Mx	2,5	Atypical Carcinoid
#5	F	47	15/01/1963	SX	<1%	T1N0Mx	1,	Typical Carcinoid
#6	M	57	06/05/1954	DX	60%	T1bN2Mx	2,3	Typical Carcinoid
#7	F	35	26/01/1976	DX	8%	T2aN0Mx	4,5	Typical Carcinoid
#8	M	58	20/09/1973	DX	2%	T1aN0Mx	1	Typical Carcinoid
#9	M	38	26/12/1966	DX	<1%	pT1aN0Mx	1,7	Typical Carcinoid
#10	F	61	15/04/1937	DX	<1-2%	pT1aN0Mx	2	Typical Carcinoid
#11	M	46	27/02/1994	DX	10%	pT1aN0Mx	2	Typical Carcinoid
#12	M	75	12/06/1936	SX	40%	pT1aN0Mx	1,5	Typical Carcinoid
#13	M	18	16/10/1974	DX	5%	pT1aN0Mx	2	Typical Carcinoid
#14	F	21	29/05/1951	DX	4%	pT1aN0Mx	1,8	Typical Carcinoid
#15	M	24	26/09/1990	DX	2%	T1bN0Mx	3	Typical Carcinoid
#16	M	69	26/10/1945	DX	5%	pT1aN0Mx	2	Typical Carcinoid
#17	F	48	03/0671966	DX	5%	pT1bN0Mx	2	Typical Carcinoid
#18	F	77	11/11/1937	SX	1%	pT1aN0Mx	1,5	Typical Carcinoid
#19	M	34	29/05/1980	DX	1%	T1N0Mx	2	Typical Carcinoid
#20	M	43	25/10/1971	DX	7%	T2aN2Mx	3,2	Typical Carcinoid
#21	F	69	19/01/1946	SX	1%	T1aN0Mx	0,15	Typical Carcinoid
#22	F	38	09/01/1977	DX	<1%	T2bN1Mx	5	Typical Carcinoid

Table 14: BC patients' generalities

For each patient, a tissue fragment was immediately frozen in liquid nitrogen under ribonuclease-free condition at the time of surgery and stored at -80 °C until total protein or DNA isolation was performed. The remaining tissue sample was collected, in accordance with the guidelines of the local committee on human research, and immediately minced in RMPI 1640 medium under sterile condition [360]. Tissues were incubated with 0.25% trypsin, 0.35% collagenase (Sigma, Milano, Italy) and 1% trypsin at 37 °C for 40/60 min, depending on the size and characteristics of each tissue sample. Cell suspensions were filtered through double layers of gauze and washed twice with serum-free RPMI 1640 (Euroclone Ltd., Wetherby, UK). Tumor cells were resuspended in RPMI 1640 10% FBS and antibiotics, seeded in 96-well culture plates ($1x10^4$ cells/well) and incubated at 37 °C in a humidified atmosphere of 5% CO_2-95% air. After approximately 18 hours, cells were treated with test substances, with further evaluation of cell viability and caspase 3/7 activation. Informed consent of the patients was obtained for disclosing clinical investigation and per- forming the *in vitro* study.

8.4 CELL VIABILITY ASSAY:

Cell viability was assessed by ATPlite assay (Perkin-Elmer, Monza, IT) and luminescence was measured with the EnVision™ 2104 Multilabel Reader (Perkin-Elmer) [361]. The CellTiter-Glo® Luminescent Cell Viability Assay is a homogeneous method to determine the number of viable cells in culture based on quantitation of the ATP present, which signals the presence of metabolically active cells and is directly proportional to the cells number present in the culture. The Assay relies on the properties of a proprietary thermostable luciferase (Ultra-GloTM Recombinant Luciferase), which generates a stable "glow-type" luminescent signal after a specific reaction, as shown in Figure 11, with a consequent light emission.

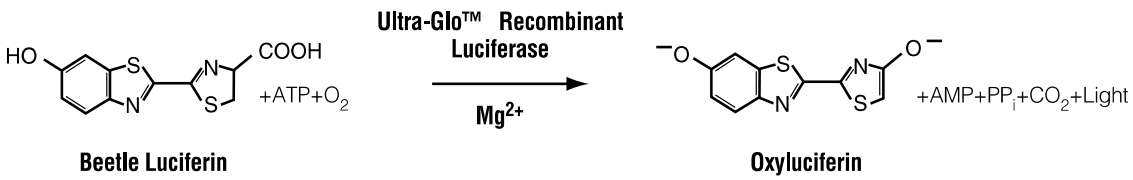


Figure 11: Mono-oxygenation of luciferin is catalyzed by luciferase in the presence of Mg2+, ATP and molecular oxygen.

Briefly, for Everolimus (100 nM) and Dinaciclib (50 nM, 100 nM) treatments, assessed on both primary human BC cultures and BC cell lines *in vitro* cell lines NCI-H720 and NCI-H727 treatments (as a single agent and/or a combination of both), the cells were seeded at 2 x 10^4 cells per well in 96-well white plates and treated with the indicated substances. Control cells were treated with the vehicle alone (0.1% DMSO). Treatments were renewed every 24 h; after incubation time (48h and/or 72h), cell viability assay was assessed adding substrate solution directly to cell culture plates. Results are expressed as mean value ± standard error of the mean (SEM) percent cell viability vs. control cells in six replicates. Regarding Dinaciclib concentration-scale (2 μM, 1 μM, 0,5 μM, 0,25 μM, 0,125 μM, 0,0625 μM, 0,03 μM, 0,0125 μM, 0,00625 μM) and combined treatments, assessed on BCs NCI-H720 and MCI-H727, the cells were seeded at 2 x 10^4 cells per well in 96-well white plates and treated with the indicated substances. Control cells were treated with the vehicle alone (0.1% DMSO). Treatments were renewed every 24 h; after incubation time (48h and 72h), cell viability assay was assessed adding substrate solution directly to cell culture plates. Results are expressed as mean value ± standard error of the mean (SEM) percent cell viability vs. control cells in six replicates.

8.5 CASPASE ASSAY:

Caspase activity was performed by using Caspase-Glo 3/7 assay (Promega, Milano, IT) according to the manufacture's instructions [362]. Luminescence was measured with the EnVision™ 2104 Multilabel Reader Multilabel Counter (Perkin-Elmer) and expressed as relative light 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 [363-364]. The Caspase-Glo® 3/7 Assay relies on the properties of a proprietary thermostable luciferase (Ultra-GloTM Recombinant Luciferase), which is formulated to generate a stable "glow-type" luminescent signal, after cell lysis and caspase cleavage of the substrate, Figure 12; luminescence is proportional to the amount of caspase activity present.

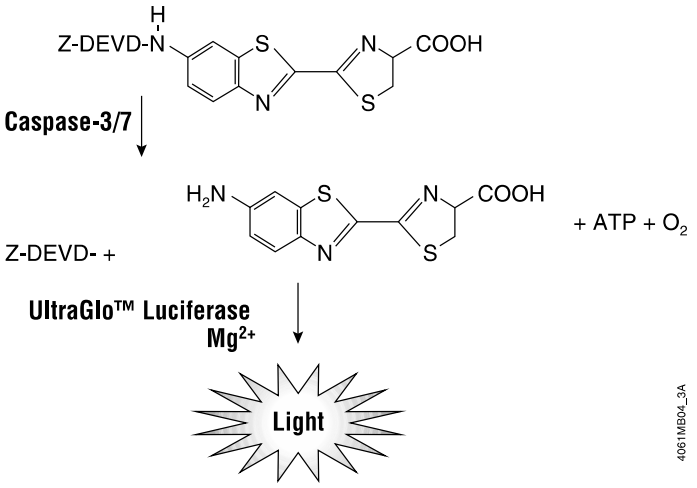


Figure 12: Caspase-3/7 cleavage of the luminogenic substrate containing the DEVD sequence. Following caspase cleavage, a substrate for luciferase (aminoluciferin) is released, resulting in the luciferase reaction and the production of light.

Briefly, the cells were seeded at 2 x 10^4 cells per well in 96-well white plates and treated with the indicate compounds. All treatments, (concentration-scale, single and/or a combination of Danicilcib/Everolimus compaunds), renewal incubation time have been assessed as described above for ATPlite Assays on both human BC primary cultures and *in vitro* BC NCI-H720 and NCI-H727 cell lines; luminescent output (Relative Light Unit, RLU) was measured. Results are expressed as mean value ± SEM percent RLU vs. control cells in six replicates.

8.6 PROTEIN ISOLATION AND WESTERN BLOT ANALYSIS

NCI-H727 and NCI-H720 cells were seeded at a cell density of 2 x 10^4 cells per ml in 75-mm plates in complete medium (10% FBS). Cells were synchronized by over night incubation in 0.1% FBS medium. The day after, cells were treated with or without indicated compounds in complete medium and than after 0-3-6-9-12-18-24-36 hours cell pellets were collected. After treatment with the test substances at different time points, cells were resuspended in sample buffer [60 mm Tris-HCl (pH 6.8), 10% glycerol, 2% sodium dodecyl sulfate] and lysed by boiling at 100 C for 5 min [(365)]. For protein isolation from BC tissues, total cell lysates deriving from homogenate frozen tissues were obtained using Tissue Raptor (Qiagen), according to the manufacturer's instructions, and dissolved in RIPA buffer (Pierce, Rockford, IL, USA); subsequently kept in ice for 30 min, and then centrifuged for 10 min [(366)]. Total protein extracts obtained from both human BC tissues and cell lines, were quantified by the BCA Protein Assay Reagent Kit (Pierce Biotechnology, Inc, Rockford, Illinnois) and 30 µg of proteins were fractionated on SDS-PAGE and transferred by electrophoresis to nitrocellulose membranes (Schleicher & Schuell Italia SRL, Milano, IT).

Protein panel profile was performed by using protein extracts and the following primary antibodies: 1:1000 rabbit $p27^{kip1}$-SER10 antibody (Epitmics, Burlingame, USA), 1:200 mouse $p27^{kip1}$ antibody (BD Trasduction Laboratories, Canada, USA), 1:1000 rabbit Cyclin D1 antibody (Abcam, Cambridge, MA, USA), 1:500 mouse CDK4 antibody (Abcam, Cambridge, MA, USA), 1:500 rabbit Cyclin E antibody (Abcam, Cambridge, MA, USA), 1:2000 rabbit CDK2 antibody (Cell Signalling, Beverly, MA, USA), 1:1000 rabbit anti-GAPDH (Cell Signalling, Beverly, MA, USA). Immunoreactive proteins were visualized by enhanced chemioluminescence (Amersham International). Quantification of the bands' intensity was performed by using the Gel Doc System with the Quantity One Software (Bio-Rad). Three independent experiments were performed; data are expressed as the ratio between protein of interest and GAPH signal intensity, expressed as percent vs. control. For time-course experiments, 0-36 h protein levels histograms were built and the Area Under the Curve (AUC) was assessed using GraphPad PRISM^6 Software.

8.7 DNA ISOLATION AND *CDKN1B* GENE DIRECT SEQUENCING

Genomic DNA (gDNA) was isolated from whole blood of available patients' samples, by using the QIAmp DNA Blood Mini Kit (Qiagen, Milano, Italy), by using the QIAamp DNA Blood Mini Kit (QIAGEN, Milano, Italy) on the QIAcube automated system (Qiagen) [367]. Somatic DNA (sDNA) was extracted from each BC frozen tissue, disrupted with Tissue Raptor (Qiagen), according to the manufacturer's instructions. sDNA from homogenate tissues was extracted with QIAmp-Micro Kit (Qiagen, Milano, IT) on the QIAcube™ automated system (Qiagen, Milano; IT). DNA was quantified by Wallac Victor™ 1420 Multilabel Counter (Perkin-Elmer). At least 100 ng of DNA were used for each application.

For *CDKN1B* gene mutation analysis, coding regions and intron-exon boundaries were amplified by Polymerase Chain Reaction (PCR) using the GeneAmp PCR System 9700 thermal cycler (Life Technologies, Milano, IT) by using specific primers, Box 3, and applying the following thermal cycling conditions: incubation at 96°C for 3 min; 35 cycles at 94°C for 30 sec, 66 °C for 1 min, and 72 °C for 1 min with a final extension at 72 °C for 7 min. Each reaction mixture was prepared using GoTaq green master mix (Promega, Milano, IT), [368].

The amplified products were purified with the QiaQuick PCR purification kit (Qiagen) and then submitted to sequencing reaction using the BigDye Terminator Cycle Sequencing Ready Reaction Kit 3.1 (Applied Biosystems), applying the following cycle profile: 96 8C for 10 s and 60 8C for 4 min (45 cycles). The samples were then purified and direct sequencing was performed, using the same primers mentioned before, on the 3130 Genetic Analyzer (Life Technologies, Milano, IT), [369].

Name	Sequence
5′UTR p27_ChIP for	5′-TGTGTCTTTTGGCTCCGAGG-3′
5′UTR p27_ChIP rev	5′-CTCCCGTTAGACACTCGCAC-3′
5′UTR p27 for	5′-GAG GAG CGG GAG GGA GGT CG-3′
5′UTR p27 rev	5′-CTAGGGCTCCCGTTAGACACT-3′
p27ex1A for	5′-CGTCAGCCTCCCTTCCACCG-3′
p27ex1A rev	5′-CTCTTCGTGGTCCACCGGGC-3′
p27ex1B for	5′-GAGCCCTAGCCTGGAGCGGAT-3′
p27ex1B rev	5′-GCGGGGCCCCAAACACATTCT-3′
p27ex2 for	5′-CTGACTATGGGGCCAACTTC-3′
p27ex2 rev	5′-GCCAGCAACCAGTAAGATCAG-3′
p27ex3A for	5′-TGAACACTGGCTAAAGATAATTGCTATTTA-3′
p27ex3A rev	5′-TGCCAGGTCAAATACCTTGTTTG-3′
p27ex3B for	5′-GACCAAAGAACACAGCACAGAGGA-3′
p27ex3B rev	5′-CTGGGGAGGGCAGTGAGGAT-3′
5′UTR p27 BglII	5′ GAG GAG CGG GAG GGA GAT CTG GGC TT 3′
5′UTR p27 NcoI	5′ GAC ACT CGC ACG TTT GCC ATG GTT CTC 3′

Box 3: Primers employed to amplify CDKN1B promoter and coding regions

8.8 IMMUNOHISTOCHEMISTRY

Immunohistochemistry (IHC) was performed, in collaboration with University of Padua and Helmöhltz Zentüm München, by using a monoclonal anti-$p27^{kip1}$ antibody (BD, Bioscences, CA, USA) and the monoclonal MIB5 antibody (Dako, Hamburg, Germany) as previously described [370-371].

9. STATISTICAL ANALYSIS

Results of cell viability and caspase activation experiments are expressed as mean value ± standard error of the mean (SEM) percent vs. control. Concerning the results of the above described assays, as well as tissue $p27^{kip1}$ index, a preliminary analysis was carried out in order to determine whether the data sets conformed to a normal distribution using both D'Agostino Pearson omnibus and Shapiro-Wilk normality tests. Student's unpaired t test and/or Mann-Whitney test were used to evaluate individual differences between means (GraphPad PRISM[6] Software). Differences were considered significant at $P < 0.05$.

For human BC primary culture multiple treatments, Everolimus and/or Dinaciclib as a single treatment and combination of both at different concentrations, differences between treatment groups for in vitro data were assessed by ANOVA (one-way) or Kruscall-Wallis, with appropriate linear contrasts, while multiple comparisons was assessed by using the Bonferroni test or the Dunn's test, depending on the data distribution if they resulted normal or not respectively. Normality test and the other ones were assessed, as previously described, by using GraphPad PRISM[6] Software. Differences were considered significant at $P < 0.05$.

As regards genetic analysis, data were summarized by calculating means and standard errors for quantitative variables and numbers and percentages for categorical variables. Everolimus response (yes/no) in primary cultures was used as grouping variable for the primary analysis in this study. For secondary analyses, sample was grouped using histology (typical/atypical) and lymph nodes disease (presence/absence). Differences between groups were compared using t-test and Chi-square, as appropriate (U-Mann Whitney for variables with not normal distribution). Logistic regression models were used to determine if there was an association between genetic mutations and Everolimus response in primary cultures. The p-values were two-sided and statistical significance was considered when $P < 0.05$. All statistical analyses were performed using STATA version 13.0 (StataCorp LP, Texas, USA) and GraphPad version 5.0 (GraphPad Software, La Jolla, CA).

10. RESULTS

10.1 CELL CYCLE CONTROL PROTEIN PROFILE IN BC CELL LINES

G_0/G_1 to S cell cycle progression phase depends on the coordinate expression of Cyclins that bind to active Cdks, which, in turn, are regulated by Cdk-Inhibitors (CdkI). We previously demonstrated that BCs cell lines resistant to m-TOR inhibitors display a reduction in Cyclin D1 levels after treatment with Everolimus, despite they continue to proliferate [273]. Therefore, we investigated whether cell cycle protein profile is different in the BC cell line sensitive to Everolimus as compared to the BC cell line resistant to Everolimus.

After starvation over night, NCI-H720 and NCI-H727 cells, representing BCs in vitro models sensitive and resistant to m-TOR inhibitors, respectively, were incubated with RPMI 1640 supplemented with 10% FBS for 0, 3, 6, 9, 12, 18, 24, 36 h. Western blot analysis for Cyclin D1 - Cdk4 (Figure 13 *A, B*), Cyclin E - Cdk2 (Figure 14 *A, B*), $p27^{kip1}$ and p-SER10 $p27^{kip1}$ (see next paragraph) were performed. We observed that the two BC cell lines display a different G_0/G_1 to S cell cycle control protein profile. Protein signal densitometry was performed by using QuantityOne software on Gel Doc System BioRad, for each time point, each protein and experiment. Time-course linear graphs (Figure 13-Figure 14 *C, D, E* and *F*) for each protein were built and Area Under the Curve (AUC) was calculated as Intensity x mm^2. Finally, AUCs were expressed as percentage vs. starting time (T0h), and shown as histograms (Figure 13-Figure 14 *G, H*).

As shown in time course linear graphs, (*Fig. 13* and *Fig. 14 C, D, E, F),* as well as in the histograms representation, (*Fig. 13* and *Fig. 14 G*, *H*), during the 0-36 h time frame resistant cells showed higher Cyclin/Cdk protein levels. Cyclin D1 (*Fig. 13 G*), Cyclin E (*Fig. 14 G*) and Cdk2 (*Fig. 14 H*) protein levels were ~2-fold in NCI-H727 as compared to NCI-H720 cells. Cdk4 protein levels in resistant cells were ~5-fold as compared to sensitive cells (Fig. 13 *H*).

Since both Cyclin D1/Cdk4 and Cyclin E/Cdk2 act as positive regulators of G_0/G_1 to S cell cycle phase transition [276] these data indicate that NCI-H727 cells display a higher transition to S phase along the 36 h time frame as well.

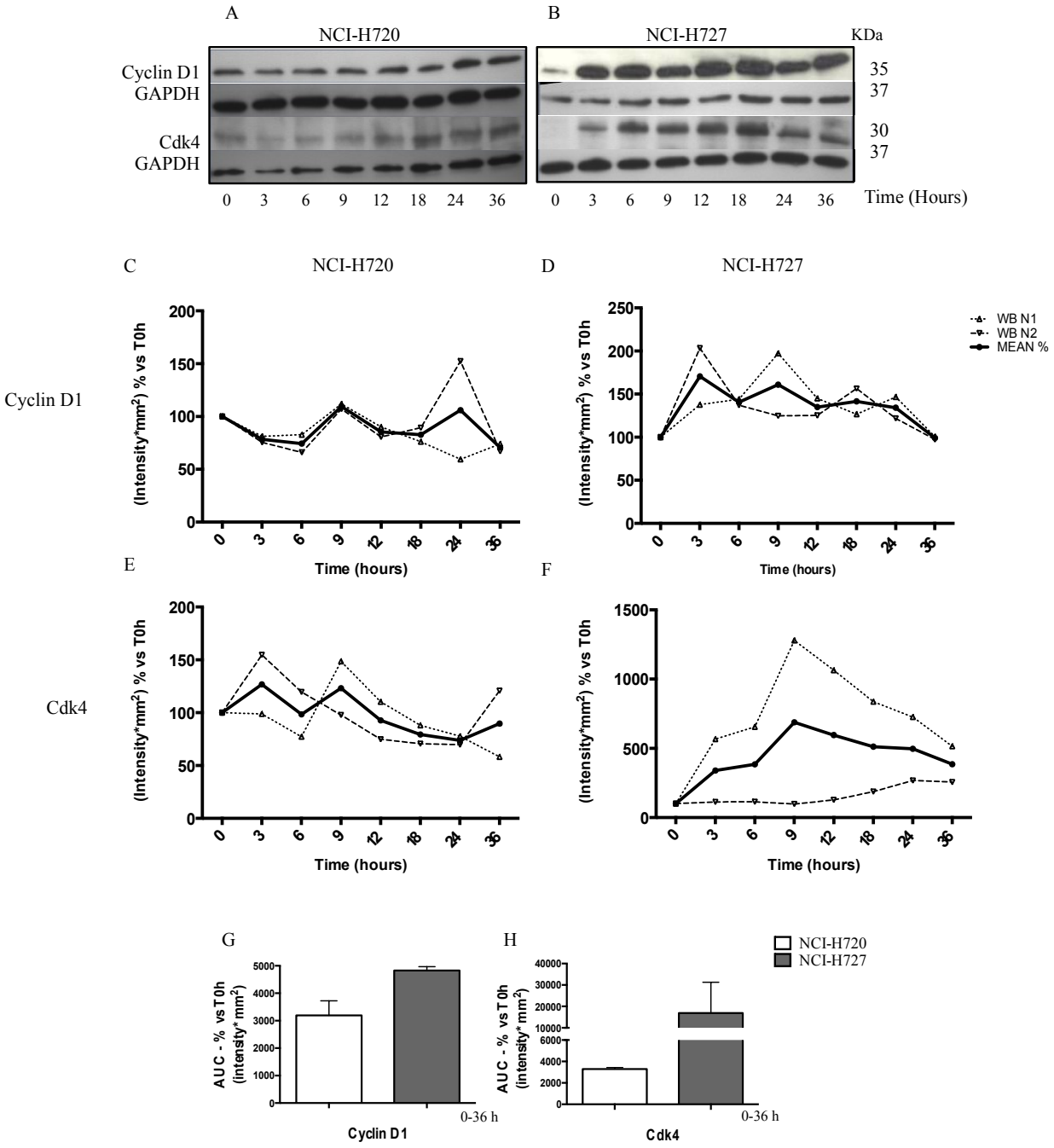


Figure 13: Time course of Cyclin D1 and Cdk4 protein expression levels in BC *in vitro* cell lines. Western blot analysis for NCI-H720 (A) and NCI-H727 (B) cells; time course (0-3-6-9-12-18-24-36 hours) of protein expression level of Cyclin D1 (C-D) and Cdk4 (E-F) in BCs expressed as Intensity x mm^2 in % vs. initial time (T0); two independent experiments are reported as dotted lines while the time course protein expression level mean value is represented as straight line; The histograms describe % AUC (Intensity x mm^2) ± SEM vs. time 0 (T0) abundance of Cyclin D1 (G) and Cdk4 (H) in NCI-H720 (white bars) and in NCI-H727 (grey bars) cells over a time frame of 36 h.

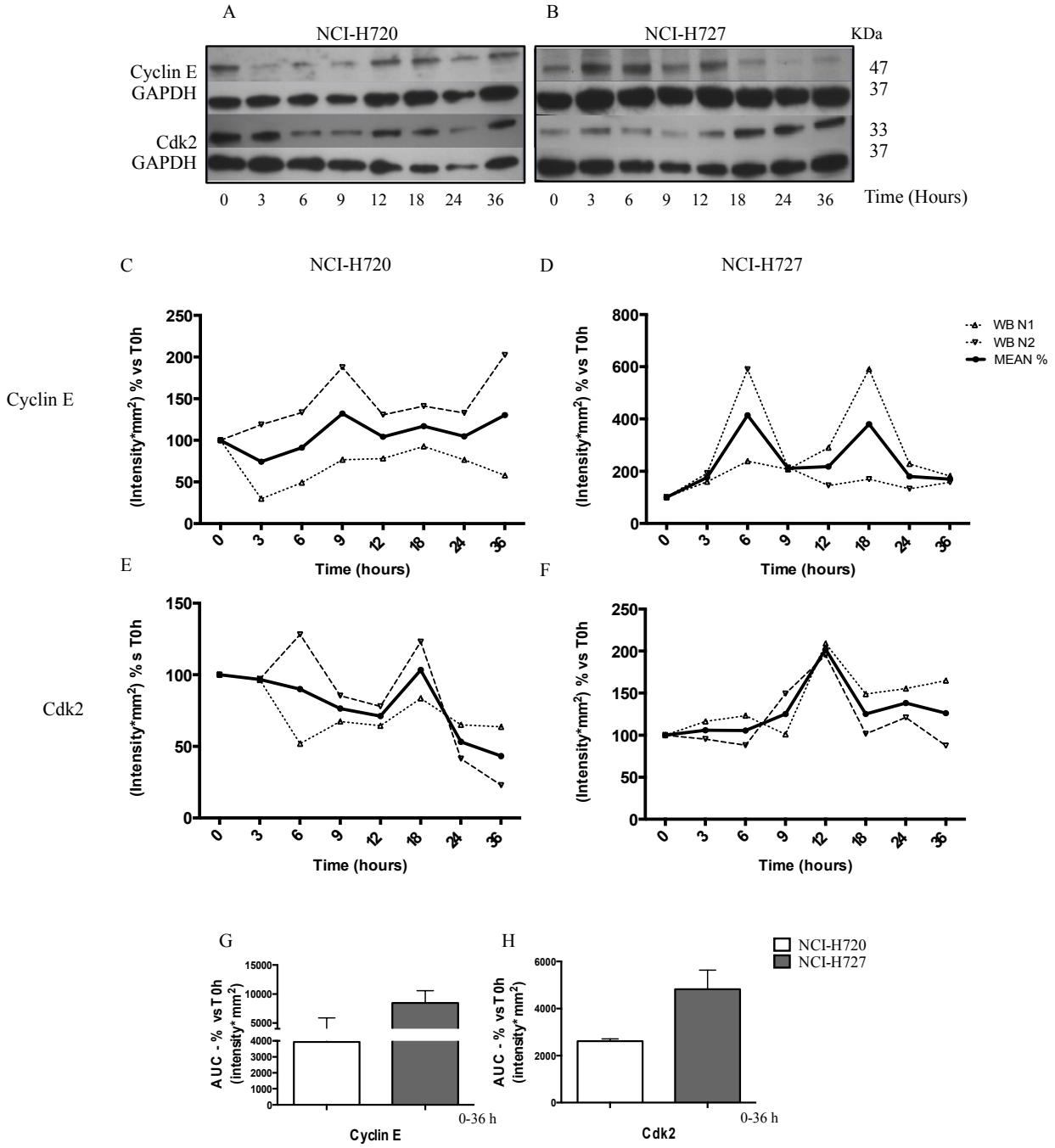


Figure 14: Time course of Cyclin E and Cdk2 protein expression levels in BC *in vitro* cell lines. Western blot analysis for NCI-H720 (A) and NCI-H727 (B) cells; time course (0-3-6-9-12-18-24-36 hours) of protein expression level of Cyclin E (C-D) and Cdk2 (E-F) in BCs expressed as Intensity x mm^2 in % vs. initial time (T0); two independent experiments are reported as dotted lines while the time course protein expression level mean value is represented as straight line; The histograms describe % AUC (Intensity x mm^2) ± SEM vs. time 0 (T0) abundance of Cyclin E (G) and Cdk2 (H) in NCI-H720 (white bars) and in NCI-H727 (grey bars) cells over a time frame of 36 h.

10.2 Cdk INIBITOR $p27^{kip1}$ AND PHOSPHO SER10 $p27^{kip1}$ PROTEIN PROFILE IN BC CELL LINES

It is well known that the activity of Cyclin/Cdk complexes is regulated by several mechanisms, including the association with a class of CdkI [278-284]. This interaction inhibits Cyclin/Cdk complexes catalytic activity, leading to a restraint in cell cycle progression. CdkI $p27^{kip1}$ plays a central role in G_0/G_1 to S phase transition, modulating Cyclin D/Cdk4 and Cyclin E/Cdk2 complexes activity, following a variety of antimitogenic signals [286-287]. Therefore, we also investigated $p27^{kip1}$ and p-SER10 $p27^{kip1}$ protein levels in the 0 – 36 h time frame, in the two BCs cell lines. As shown in *Fig. 15 (C, G)*, total $p27^{kip1}$ levels were ~5-fold in resistant cells as compared to sensitive cells, *Fig. 15 (D, G)*. On the contrary, p-SER10 $p27^{kip1}$ levels were slightly higher in sensitive, *Fig. 15 (E, H),* as compared to resistant BC cells, *Fig. 15 (F, H)*.

As assessed before, after starvation over night, NCI-H720 and NCI-H727 cells were incubated with RPMI 1640 supplemented with 10% FBS for 0, 3, 6, 9, 12, 18, 24, 36 h. Subsequently total protein were extracted and Western blot analysis for $p27^{kip1}$ and p-SER10 $p27^{kip1}$ (Figure 15 *A, B*) were performed. As for G_0/G_1 to S phase transition protein levels expression also for CdkI $p27^{kip1}$ different protein expression profiles have been observed. Protein signal densitometry was performed by using QuantityOne software on Gel Doc System BioRad, for each time point, each protein and experiment. Time-course graphs (Figure 15 *C, D, E and F*) for each protein were built and Area Under the Curve (AUC) was calculated as Intensity x mm^2. Finally, AUCs were expressed as percentage vs. starting time (T0h), and shown as histograms (Figure 15 *G, H*).

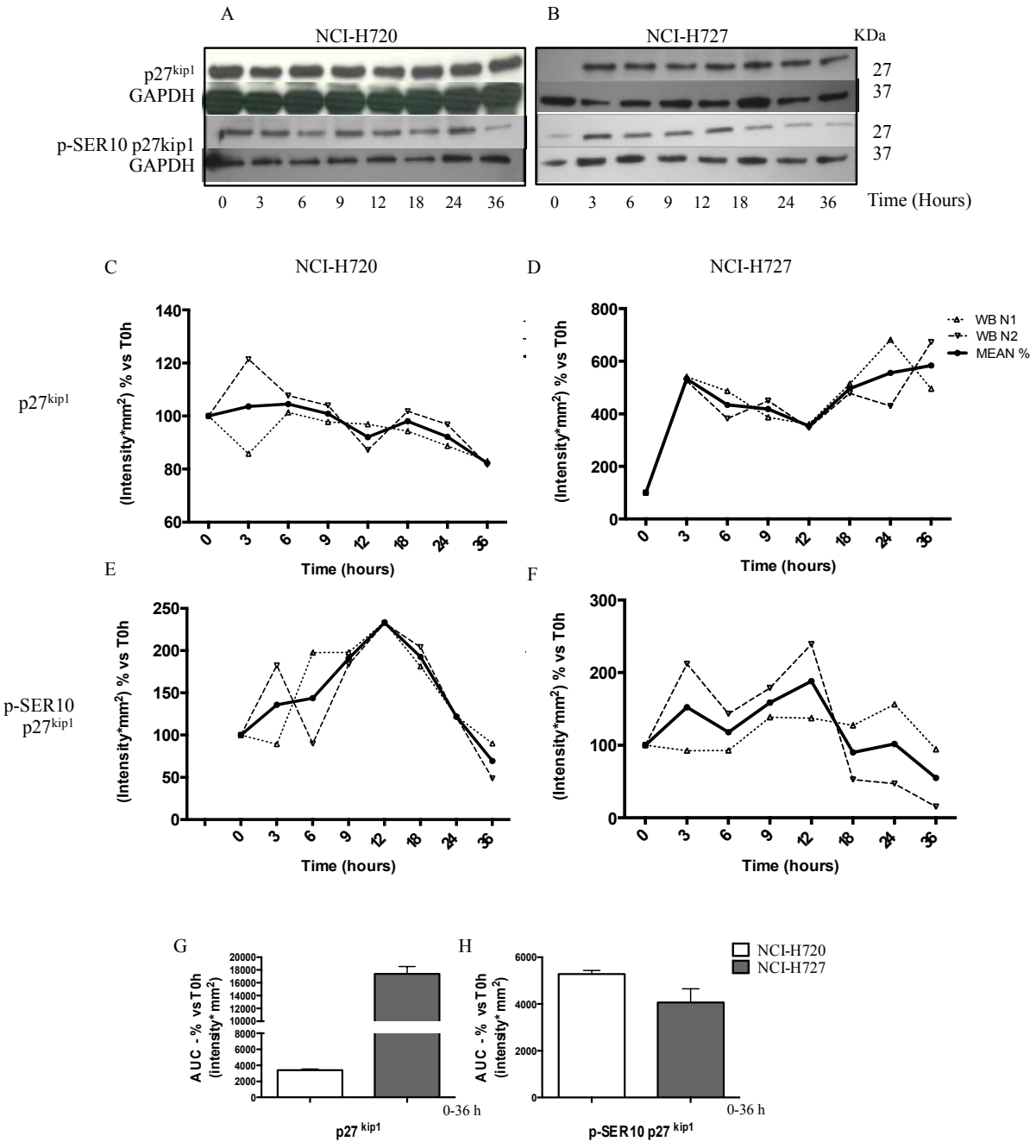


Figure 15: Time course of $p27^{kip1}$ and p-SER10 $p27^{kip1}$ protein expression levels in BC *in vitro* cell lines. Western blot analysis for NCI-H720 (A) and NCI-H727 (B) cells; time course (0-3-6-9-12-18-24-36 hours) of protein expression level of $p27^{kip1}$ (C-D) and p-SER10 $p27^{kip1}$ (E-F) in BCs expressed as Intensity x mm^2 in % vs. initial time (T0); two independent experiments are reported as dotted lines while the time course protein expression level mean value is represented as straight line; The histograms describe % AUC (Intensity x mm^2) ± SEM vs. time 0 (T0) abundance of $p27^{kip1}$ (G) and p-SER10 $p27^{kip1}$ (H) in NCI-H720 (white bars) and in NCI-H727 (grey bars) cells over a time frame of 36 h.

10.3 CELL CYCLE CONTROL PROTEIN PROFILE IN BC CELL LINES AFTER 100 nM EVEROLIMUS TREATMENT

To investigate whether treatment with m-TOR inhibitors could affect protein expression levels of analyzed Cyclins/Cdks and CdkI, the same analysis was performed under treatment with 100 nM Everolimus. After starvation over night, NCI-H720 and NCI-H727 cells were incubated with RPMI 1640 supplemented with 10% FBS supplemented with 100 nM Everolimus for 0, 3, 6, 9, 12, 18, 24, 36 h. Western blot analysis for Cyclin D1 - Cdk4 (Figure 16 A, B), Cyclin E - Cdk2 (Figure 17 A, B), p27kip1 and p-SER10 p27kip1 (see next paragraph) were performed. Protein signal densitometry time courses (Figure 16-Figure 17 C, D, E and F) and AUC (Figure 16-Figure 17 G, H) histograms construction were performed as described above.

Comparing the protein levels observed in both BCs, after 36 hours of 100 nM Everolimus treatment, Cyclin D1 and Cdk4 levels resulted quite similar in resistant, (*Fig. 16 D, F and G, H*), and in sensitive BCs, (*Fig. 16 C, E and G, H*), while, Cyclin E and Cdk2 slightly differs each others. In particular, Cyclin E levels were ~1.6-fold higher in resistant cells, (*Fig. 17 D, G),* as compared to sensitive ones, (*Fig. 17 C, G*), while Cdk2 protein levels were slightly lower in resistant BCs, NCI-H727, as compared to sensitive ones the NCI-H720 (*Fig. 17 E, F, H*).

Here we have just described how Everolimus can affect on G_0/G_1 to S cell cycle phase transition regulators, protein levels expression, in the two BCs analysed in this study; Really, to better understand the impact of m-TOR inhibitor treatments, we have also compared Cyclin/Cdk complexes and $p27^{kip1}$/p-SER10 $p27^{kip1}$ protein expression levels before and after treatment with 100 nM Everolimus in the same analysis; (see paragraph 9.5).

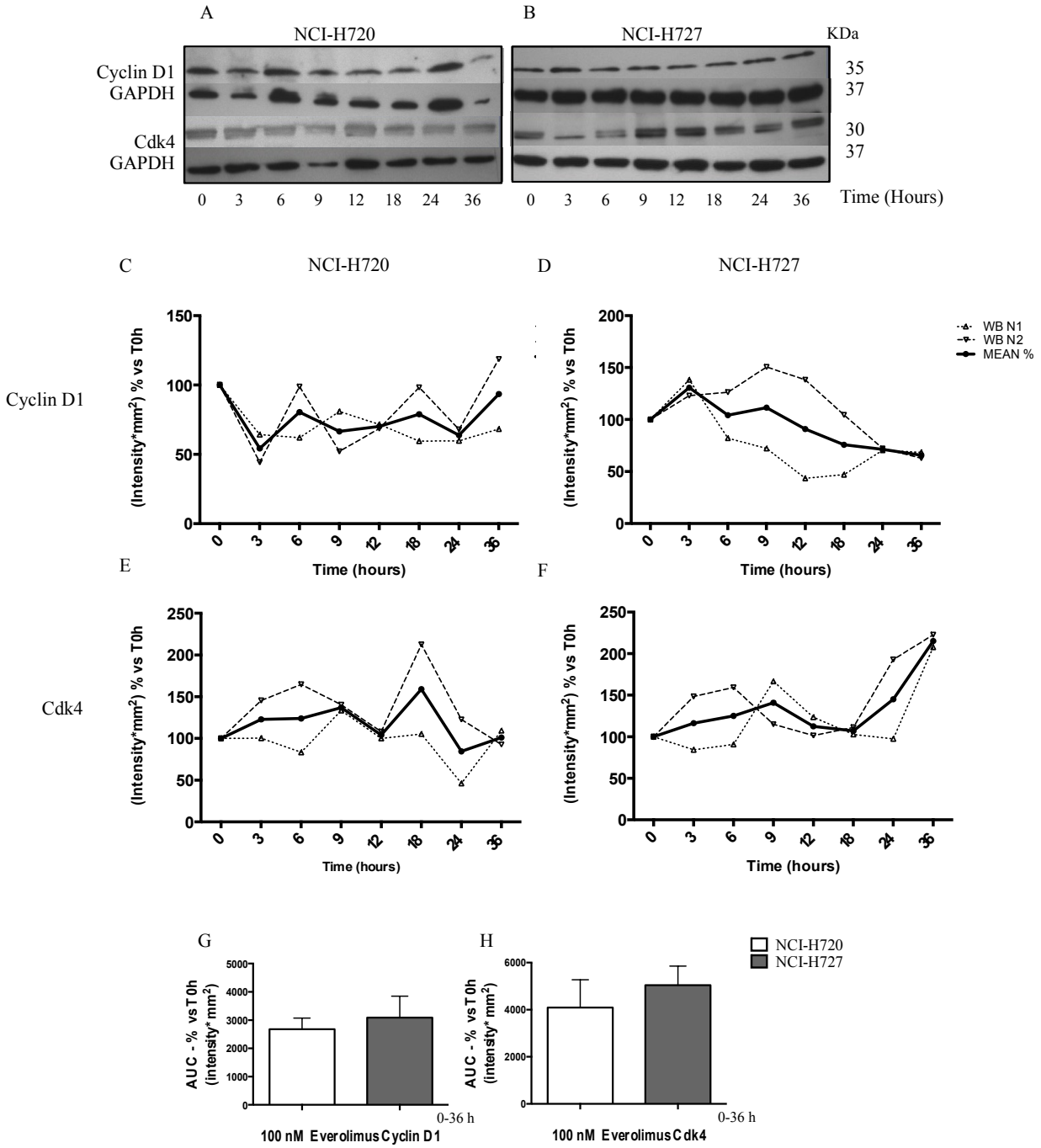


Figure 16: Time course of Cyclin D1 and Cdk4 protein expression levels in BC *in vitro* cell lines, after treatment with 100 nM Everolimus. Western blot analysis for NCI-H720 (A) and NCI-H727 (B) cells; time course (0-3-6-9-12-18-24-36 hours) of protein expression level of Cyclin D1 (C-D) and Cdk4 (E-F) in BCs expressed as Intensity x mm^2 in % vs. initial time (T0); two independent experiments are reported as dotted lines while the time course protein expression level mean value is represented as straight line; The histograms describe % AUC (Intensity x mm^2) ± SEM vs. time 0 (T0) abundance of Cyclin D1 (G) and Cdk4 (H) in NCI-H720 (white bars) and in NCI-H727 (grey bars) cells over a time frame of 36 h.

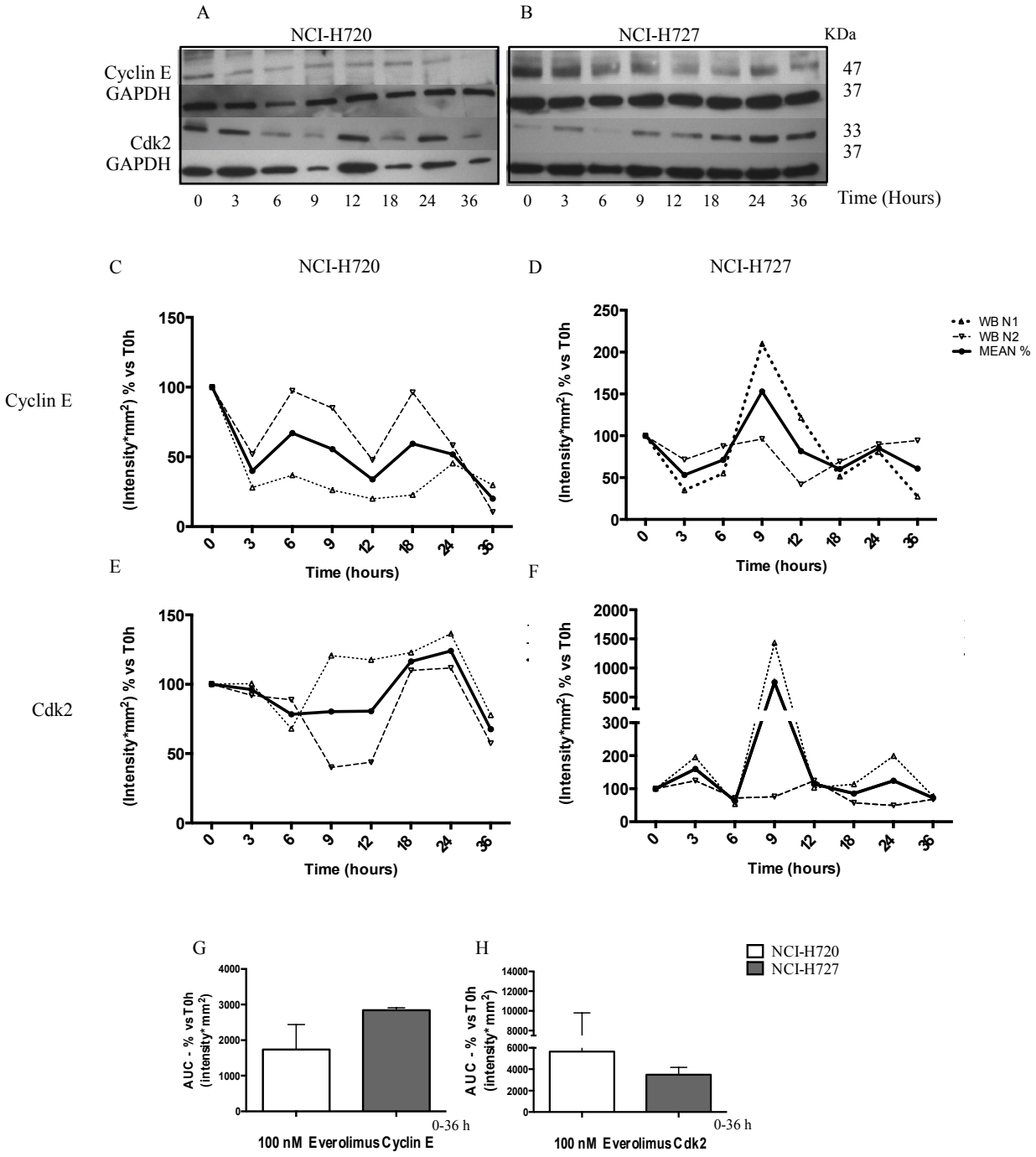


Figure 17: Time course of Cyclin E and Cdk2 protein expression levels in BC *in vitro* cell lines, after treatment with 100 nM of Everolimus. Western blot analysis for NCI-H720 (A) and NCI-H727 (B) cells; time course (0-3-6-9-12-18-24-36 hours) of protein expression level of Cyclin E (C-D) and Cdk2 (E-F) in BCs expressed as Intensity x mm^2 in % vs. initial time (T0); two independent experiments are reported as dotted lines while the time course protein expression level mean value is represented as straight line; The histograms describe % AUC (Intensity x mm^2) ± SEM vs. time 0 (T0) abundance of Cyclin E (G) and Cdk2 (H) in NCI-H720 (white bars) and in NCI-H727 (grey bars) cells over a time frame of 36 h.

10.4 CDK INIBITOR $p27^{kip1}$ AND PHOSPHO SER10 $p27^{kip1}$ PROTEIN PROFILE IN BCs CELL LINES AFTER 100 nM EVEROLIMUS TREATMENT

As assessed before, after starvation over night, NCI-H720 and NCI-H727 cells were incubated with RPMI 1640 supplemented with 10% FBS for 0, 3, 6, 9, 12, 18, 24, 36 h and 100 nM Everolimus. Subsequently total protein were extracted and Western blot analysis for p27kip1 and p-SER10 p27kip1 were performed, Figure 18.

As concerns $p27^{kip1}$ and p-SER10 $p27^{kip1}$ protein levels, after treatment with 100 nM Everolimus for 0 – 36 h we found similar levels in the two BC cell lines, as shown in *Fig. 18*.

As for Cyclin /Cdk complexes as well for $p27^{kip}$/p-SER10 $p27^{kip1}$ in this section, we have generally described how Everolimus can affect $p27^{kip}$ protein levels expression in NCI-H727 and NCI-H720 BC cell lines; Subsequently, to evaluate the impact of m-TOR inhibitor treatments on this CdkI, we have also compared protein expression levels observed before and after treatment (100 nM Everolimus) in the same analysis; (see paragraph 9.5).

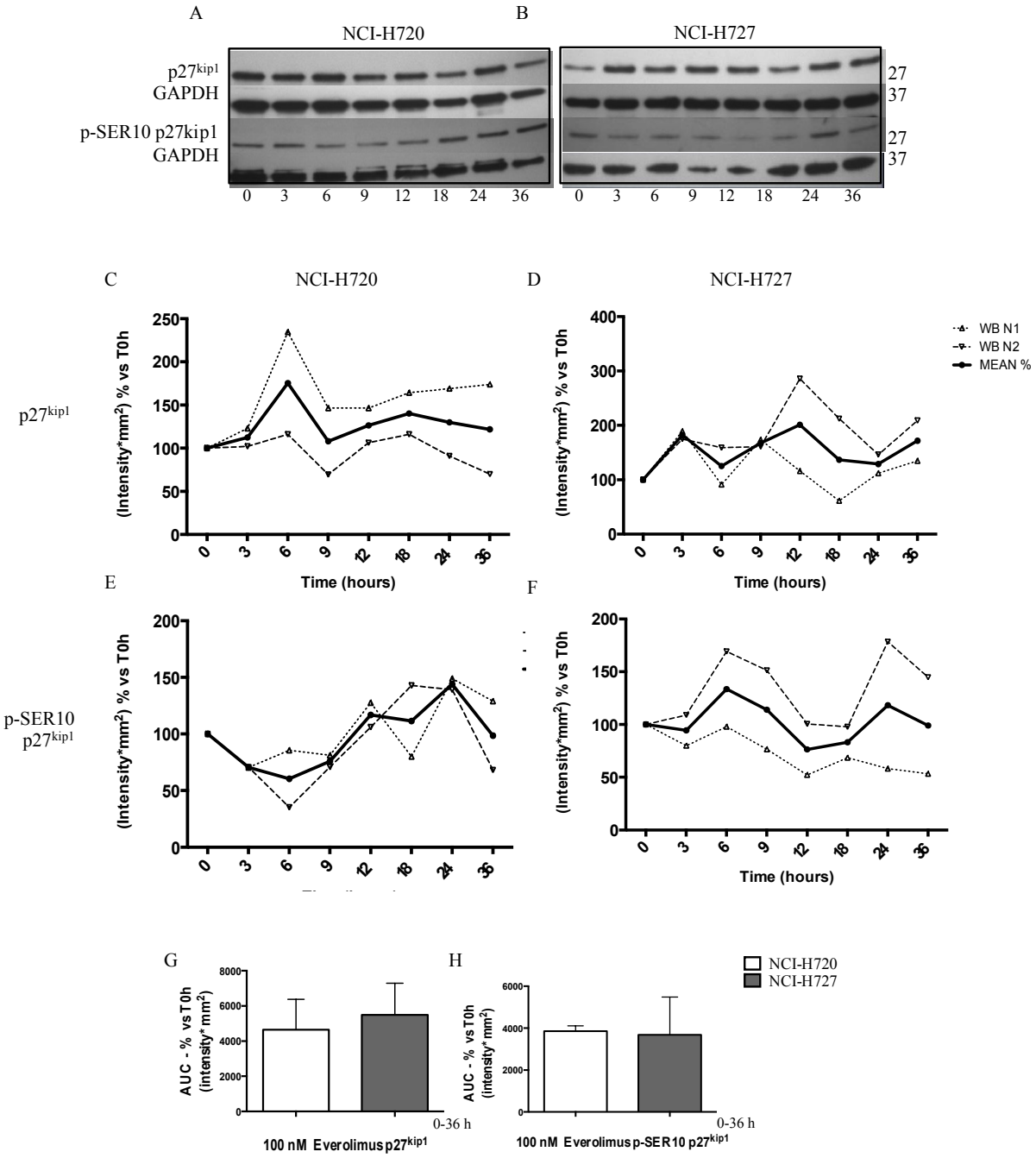


Figure 18: Time course of $p27^{kip1}$ and p-SER10 $p27^{kip1}$ protein expression levels in BC *in vitro* cell lines, after treatment with 100 nM Everolimus. Western blot analysis for NCI-H720 (A) and NCI-H727 (B) cells; time course (0-3-6-9-12-18-24-36 hours) of protein expression level of $p27^{kip1}$ (C-D) and p-SER10 $p27^{kip1}$ (E-F) in BCs expressed as Intensity x mm^2 in % vs. initial time (T0); two independent experiments are reported as dotted lines while the time course protein expression level mean value is represented as straight line; The histograms describe % AUC (Intensity x mm^2) ± SEM vs. time 0 (T0) abundance of $p27^{kip1}$ (G) and p-SER10 $p27^{kip1}$ (H) in NCI-H720 (white bars) and in NCI-H727 (grey bars) cells over a time frame of 36 h.

10.5 COMPARISON OF CYCLIN/CDK, TOTAL/p-SER10 $p27^{kip1}$ BEFORE AND AFTER 100 nM EVEROLIMUS TREATMENT IN BCs

In order to evaluate the impact of Everolimus treatment on cell cycle proteins till now analyzed, we compared Cyclin/Cdk complexes and CdkI protein expression levels observed without or with 100 nM Everolimus treatment in each BC cell line.

Results are represented as histogram (Figure 19) of 0-36 h AUC analysis, in % versus the initial time (T0h). All the AUC data, till now reported, are shown in Figure 19.

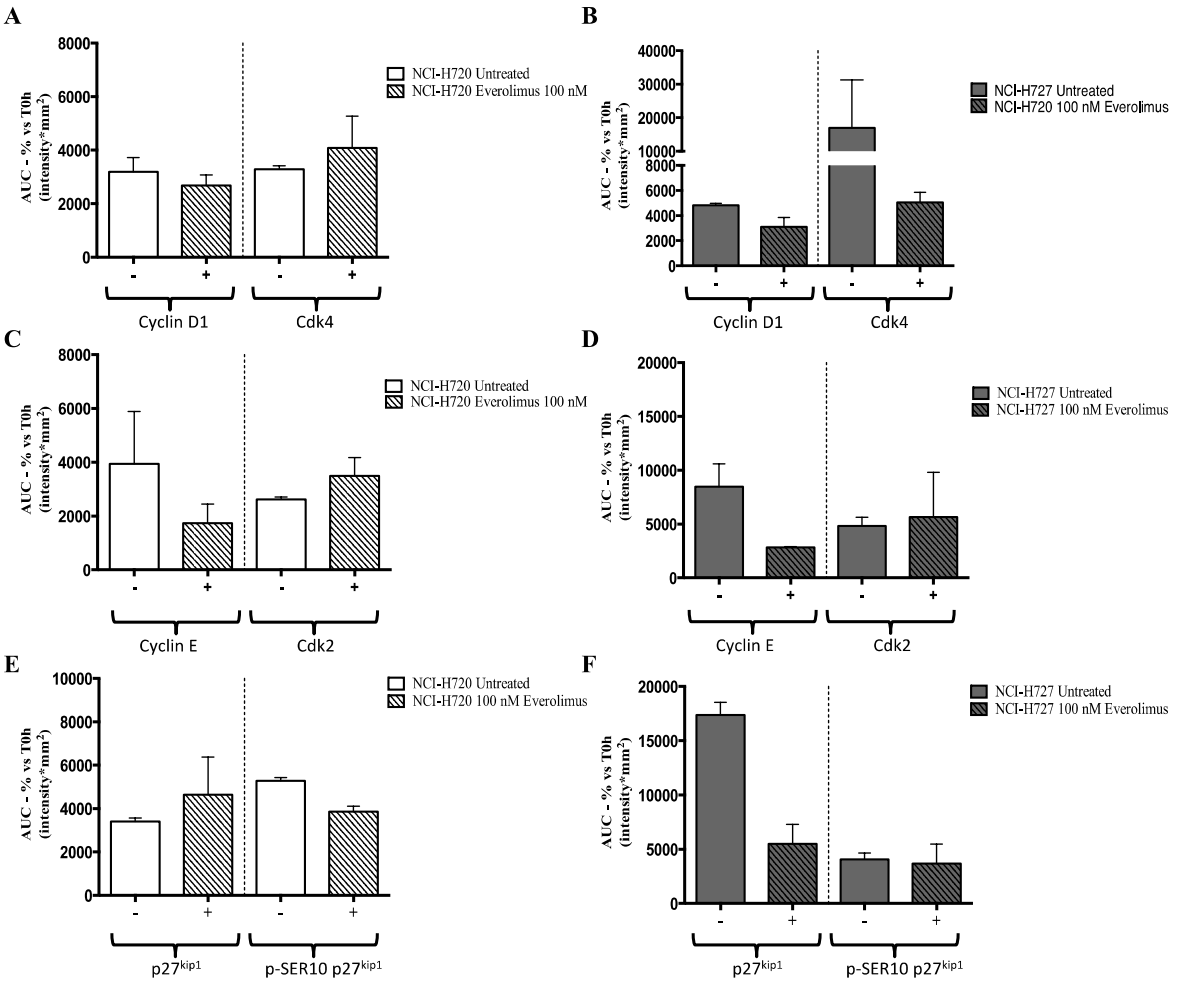


Figure 19: AUC (intensity * mm^2) expressed in % versus the initial time (T0) and represented as histograms, of G_0/G_1 to S phase protein expression levels and CdkI $p27^{kip1}$ before and after 100 nM of Everolimus treatment in BCs, NCI-H720 and NCI-H727.

	NCIH-720						NCIH-727					
	Everolimus						*Everolimus*					
	-			+			-			+		
	WB N1	WB N2	**MEAN**	WB N1	WB N2	**MEAN**	WB N1	WB N2	**MEAN**	WB N1	WB N2	**MEAN**
Cyclin D1	2820	3569	3194	2397	2957	2677	4923	4727	4825	2552	3626	3089
Cdk4	3197	3381	3290	3256	4926	4091	27080	6764	16922	4472	5615	5044
Cyclin E	2560	5313	3936,5	1236	2233	1735	9959	6955	8457	2794	2890	2842
Cdk2	2257	2681	2619	3976	3019	3497	5395	4250	4822	8594	2720	5657
Total p27^{kip1}	3303	3514	3404	5869	3425	4647	18202	16548	17375	4219	6764	5492
p-SER 10 p27^{kip1}	5384	5174	5278	4023	3677	3854	4478	3661	4070	2402	4952	3677

*0-36h AUC (Intensity*mm^2) results are expressedin % vs T 0h*

Table 15: AUC 0-36h protein expression levels of Cyclin, Cdk and p27^{kip1} observed with or without 100 nM Everolimus in BC cell lines.

As shown in Figure 19, in sensitive BC cells, the NCI-H720, treatment with 100 nM Everolimus slightly reduced Cyclin D levels and induced a ~1.2 fold increase in Cdk4 levels (Fig. 19 A). Similarly, treatment with 100 nM Everolimus caused a ~2.2 fold reduction in Cyclin E levels and a ~1.3 fold increase in Cdk2 levels (Fig. 19 C). On the contrary, treatment with 100 nM Everolimus determined a ~1.4 fold increase in p27kip1 levels and a ~1.4 fold reduction in p-SER10 p27kip1 levels (Fig. 19 E). In resistant BC cells, the NCI-H727, treatment with 100 nM Everolimus determined a ~1.6 fold reduction in Cyclin D levels and a ~3.3 fold decrease in Cdk4 levels (Fig. 19 B). Similarly, treatment with 100 nM Everolimus caused a ~3 fold reduction in Cyclin E levels and a ~1.2 fold increase in Cdk2 levels (Fig. 19 D). As concerns p27kip1 and p-SER10 p27kip1 protein levels, treatment with 100 nM Everolimus determined a ~3.2 fold decrease in p27kip1 levels; p-SER10 p27kip1 levels were similar before and after treatment (Fig. 19 F).

In BCs sensitive to m-TOR inhibitors treatments, despite both Cyclins D and E are reduced by treatment with Everolimus their Cdks are induced, suggesting that the G_0/G_1 cell cycle arrest Everolimus-mediated, is likely due to other mechanisms that excluded Cyclin/Cdk signal pathway, (i.e. p21/p53 pathway). As attended Total p27^{kip1} is induced while phosphorylation on SER10 is reduced by treatments, so that it can mediate G_0/G_1 cells restrain.

In BCs resistant to m-TOR inhibitors treatments, even though Cyclin D - Cdk4 and Cyclin E are reduced by treatments with Everolimus, a plausible G_0/G_1 cell cycle arrest has not been observed, leading to m-TOR inhibitors resistance. This could be likely due to an impaired p27^{kip1} function, overexpressed in NCI-H727 BCs as compared to NCI-H72o BCs, probably resulting in a higher sensitivity to treatment with Everolimus and the cells escape the G_0/G_1 arrest, leading to the resistance mechanisms.

10.6 RESPONSIVENESS TO EVEROLIMUS TRATMENTS OF 22 HUMAN BC PRIMARY COLTURES

The responsiveness to mTOR inhibitors of 22 BC human primary cultures, 13 males and 9 females; age = 49.6 ± 3.8 years; median = 47.5; clinical characteristics are shown in Table 14 (for each patient, BC tissues were divided in two portions: one was immediately frozen and stored at -80 °C until total protein or DNA isolation were performed. The remaining tissue was used to perform primary cultures as previously described), have been evaluated in terms of cell viability variation and caspase 3/7 activation, with or without 100 nM Everolimus treatment [(273)]. We defined as *sensitive* those primary cultures displaying a significant reduction in cell viability ($P < 0.05$) and a significant increase in caspase activation ($P < 0.05$) after treatment with Everolimus. On the contrary, we defined as *resistant* primary cultures in which cell viability and caspase activation were not significantly affected by treatment with Everolimus (Table 16).

#paz	Responce To Everolimus	Viability % vs CT	Increase/decrease Vs % CT (=100)	P value	Ststistics l analysis
#1	Resistant	101,02 %	+ 1,02 %	0,7879	Mann-Whitney U
#2	Resistant	110,11 %	+ 10,11 %	0,4740	Mann-Whitney U
#3	Sensitive	73,75 %	- 26,25 %	0,0001	Mann-Whitney U
#4	Resistant	101,26 %	+ 1,26%	0,7879	Mann-Whitney U
#5	Sensitive	72,65 %	- 27,45 %	0,0001	Mann-Whitney U
#6	Sensitive	80,28 %	- 19,72 %	0,0022	Mann-Whitney U
#7	Sensitive	49,99 %	- 50,01 %	0,0001	Mann-Whitney U
#8	Sensitive	83,78 %	- 16,22 %	0,0022	Mann-Whitney U
#9	Sensitive	85,88 %	- 14,12 %	0,0022	Mann-Whitney U
#10	Resistant	107,12 %	+ 7,12 %	0,4740	Mann-Whitney U
#11	Sensitive	98,24 %	- 1,76 %	0,7879	Mann-Whitney U
#12	Resistant	101,86 %	+ 1,86 %	0,7879	Mann-Whitney U
#13	Sensitive	73,72 %	- 26.28 %	0,0001	Unpaired T-test with same SD
#14	Sensitive	72,29 %	- 27.71 %	0,0022	Mann-Whitney U
#15	Resistant	118,03 %	+ 18,03 %	0,0411	Mann-Whitney U
#16	Sensitive	79,11 %	- 20.89 %	0,0317	Mann-Whitney U
#17	Sensitive	83,61 %	- 16,39 %	0,0022	Mann-Whitney U
#18	Resistant	107,51 %	+ 7,51 %	0,4740	Mann-Whitney U
#19	Resistant	118,36 %	+ 18,36 %	0,7143	Mann-Whitney U
#20	Resistant	85,98 %	- 14,02 %	0,0952	Mann-Whitney U
#21	Resistant	78,54 %	- 21,46 %	0,2381	Mann-Whitney U
#22	Resistant	100 %	+ 0,00 %	0,6667	Mann-Whitney U

P Value significance: P = P < 0,05 - P** < 0,005 - P*** = P < 0,0001*

Table 16: Responsiveness to Everolimus treatment in 22 human BC primary coltures.

On this basis, we identified as sensitive 11 BC primary cultures and as resistant 11 BC primary. As shown in Figure 20A, in the sensitive group (white bars), Everolimus significantly reduced cell viability (-21% vs. control untreated cells; $P < 0,0001$) while in the resistant BC primary cultures (grey bars) treatment with Everolimus did not significantly affect cell viability.

Subsequently, in order to verify whether cell viability reduction was due to the activation of apoptosis, caspase 3/7 activity assay was performed. As shown in Figure 20 B, in the sensitive group (white bars), treatment with Everolimus induced a significant increase in caspase 3/7 activation (+76,5 vs. control untreated cells; % P = 0,0421). On the contrary, treatment with Everolimus protected resistant BC primary cultures (grey bars) from apoptosis, which was significantly reduced (- 29% vs. control untreated cells; P>0,001).

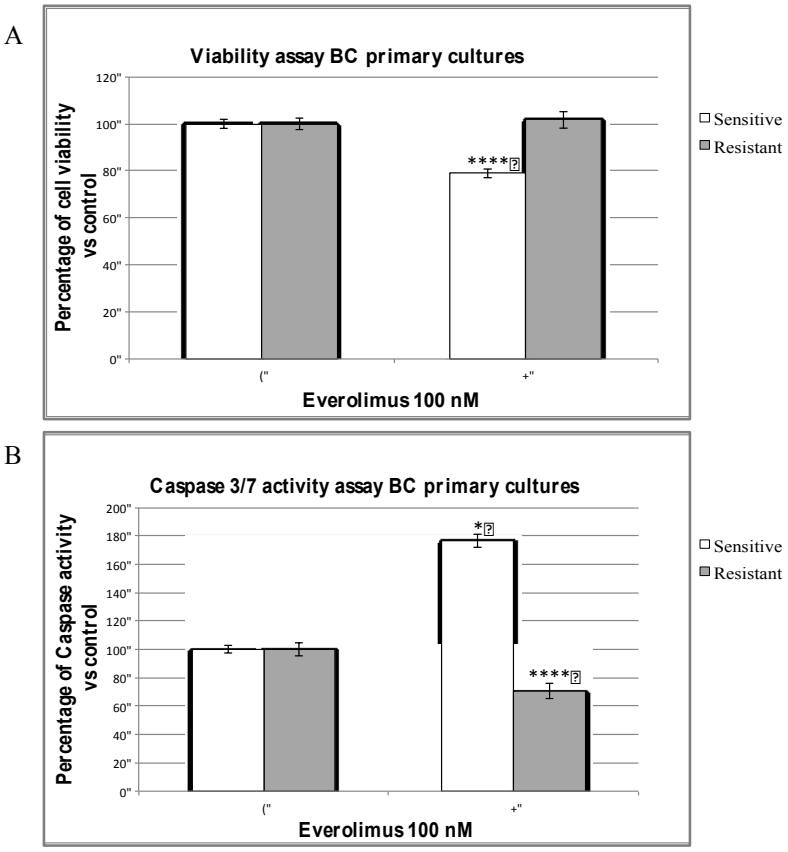


Figure 20: Cell Viability Assay (A) and caspase activation Assay (B) of BC primary cultures

Data regarding statistical analysis and resulting significance, performed on human primary BC cultures responsiveness to 100 nM Everolimus, are reported in the following table (Table 17)

	Cell Viability				*Caspase activation*			
	SENSITIVE		*RESISTANT*		*SENSITIVE*		*RESISTANT*	
	CT	*E*	*CT*	*E*	*CT*	*E*	*CT*	*E*
MEAN	100	78,93	100	101,74	100	176,52	100	70,71
ST. DEV (σ)	11,25	11,02	19,61	27,16	13,61	23,6	31,84	34,08
SEM	1,902	1,862	2,597	3,566	2,572	4,46	4,973	5,323
P value	P<0,0001 (****)		P = 0.5897		P = 0,0421 (*)		P<0,0001 (****)	

CT = control, E = Everolimus 100 nM

Table 17: BC primary cultures statistical analysis referred to Responsiveness to Everolimus treatment.

10.7 p27^{kip1} INDEX IN HUMAN BC TISSUES

Total proteins were extracted and quantified from each frozen BC tissue; with 30 μg of protein a Western blot analysis for p27kip1 and p-SER10 p27kip1 was assessed (Figure 21 A, B). Protein level quantification was performed by densitometry analysis and results of each tissue were represented in a box-plot distribution (Figure 21 C, D, E) and expressed as Intensity x mm2 in %. Two cell lines, Hela cells (CT1) and MCF7 cells (CT2), have been used as control for protein antibody detection, as suggested from datasheets.

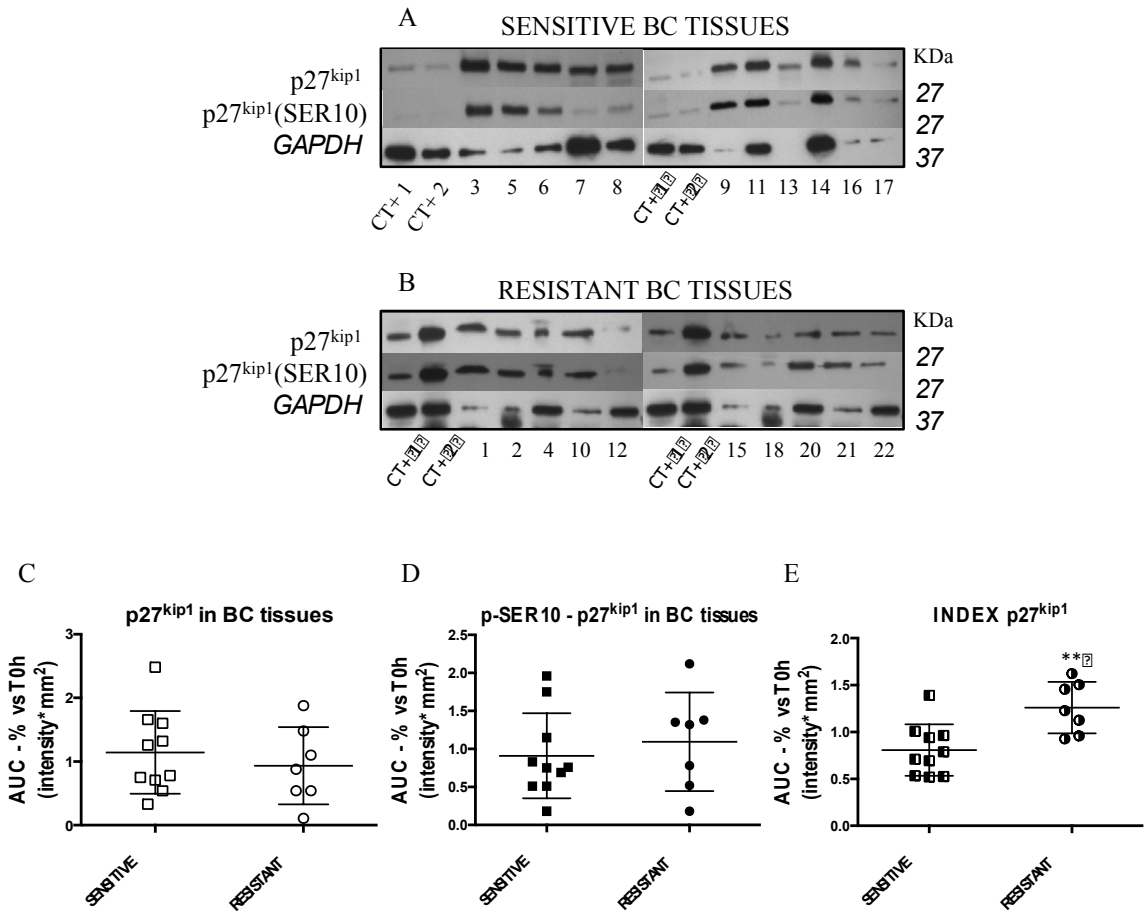


Figure 21: p27^{kip1}/phospho (SER10) p27^{kip1} protein expression in human BC tissues, western blot analysis of Sensitive BC tissues (A), Resistant BC tissues (B), Box Plot (Intensity*mm^2) p27^{kip1}(C), phospho (SER10) p27^{kip1} (D) and p27^{kip1} INDEX (E).

Sensitive BC tissues displayed ~1.2- fold higher p27kip1 total protein levels as compared to resistant BC tissues (Figure 21 C). On the contrary, sensitive BC tissues displayed ~1.2- fold lower p27kip1 p-SER10 levels as compared to resistant BC tissues (Figure 21D). We then calculated p27kip1 index (Figure 21E), obtained as the ratio between p-SER10 $p27^{kip1}$ and total $p27^{kip1}$ protein levels for each sample, assessed on each BC tissue and expressed as Intensity*mm^2 normalized versus GAPDH protein control levels expression. We found that human sensitive BC primary tissues display a significantly lower $p27^{kip1}$ index as compared to human resistant BC primary tissues.

Data regarding statistical analysis and resulting significance, performed on $p27^{kip1}$, p-SER10 $p27^{kip1}$ and $p27^{kip1}$ index protein expression levels observed on human primary BC tissues after a Western blot analysis, are reported in the following table (Table 18).

	$p27^{kip1}$		*p-SER10-$p27^{kip1}$*		*Index $p27^{kip1}$*	
	Sensitve	**Resistant**	**Sensitve**	**Resistant**	**Sensitve**	**Resistant**
MEAN	1,143	0,9329	0.9090	1,093	0,8080	1,260
ST. DEV. (σ)	0,6504	0,6073	0,5599	0,6474	0,2749	0,2737
SEM	0,2057	0,2295	0,1771	0,2447	0,0869	0,1034
KS NORMALITY TEST	Yes	Yes	Yes	Yes	Yes	Yes
UNPAIRED t-TEST (R vs. S)	**P** = 0,5111		**P** = 0,5410		**P**** = 0,0045	

Table 18: BC primary tissues statistical analysis referred to $p27^{kip1}$, p-SER10 $p27^{kip1}$ and $p27^{kip1}$ index protein expression levels of BC tissues.

According to what we have seen in BC cell lines, human BC primary tissues resistant to m-TOR inhibitors treatments, shown an higher $p27^{kip1}$ index as compared to sensitive ones; nevertheless, $p27^{kip1}$ is expressed at high levels but is less activated, as demonstrated by the low p-SER10 phosphorylated protein levels detected, in human BC sensitive tissues as compared to the resistant human BC tissues.

10.8 SOMATIC AND GERM-LINE *CDKN1B* VARIATIONS IN HUMAN BRONCHIAL CARCINOIDS

To further investigate the role played by the Cdk inhibitor $p27^{kip1}$, generally in BC tumors behavior and moreover in their responsiveness and/or resistance to mTOR inhibitors treatment, somatic and germ-line DNA were extracted from a piece of each human BC primary tissue (22 tissues samples, clinical characteristics are shown in Table 14; responsiveness to Everolimus: 11 Sensitive and 11 Resistant, data shown in (Table 16) and from available peripheral lymphocytes (14 blood samples, paz. #1, 2, 3, 4, 6, 7, 9, 11, 13, 14, 15, 16, 17, 18, clinical characteristics are shown in Table 14; responsiveness to Everolimus: 9 Sensitive and 5 Resistant, data shown in (Table 16) respectively, and a sequencing analysis for coding regions and intron-exon boundaries of *CDKN1B* gene was assessed. In addition, it has been also performed the same genetic analysis on the DNA extracted from the two BCs used for the study, the NCI-H720 and the NCI-H727, as they are representative of our in vitro model of BCs cells *Sensitive* and *Resistant* to m-TOR inhibitors treatment respectively.

Overall, among genomic and/or somatic and/or cellular CDKNIB gene, four different genetic variations have been detected: three SNPs rs34330 - rs2066862 – rs73330, and a 4 bp deletion, rs77445456; generality are reported in Table 19 while electropherograms are shown in Figure 22 (rs34330), Figure 23 (rs2066862), Figure 24 (rs73330) and Figure 25 (rs774454456);

SNPs −79C>T (rs34330), and 326T>G (V109G, rs2066827) are potentially functional, as previously reported [316]; in effect, epidemiological studies have found a significant association between V109G (rs2066827) and prostate cancer, breast cancer and oral squamous cell carcinoma [317], while, there are some evidences regarding the -79 T>C (rs34330) and the insurgence of papillary thyroid carcinoma [317]. Noteworthy, rs77445456 -26AGAG deletion has been recently found in an acromegaly patient [368], and has been correlated with a reduction in $p27^{kip1}$ mRNA levels, due to an altered secondary structure, which induces a reduced *CDKN1B* 5'UTR region transcriptional activity in vitro [368]. Moreover, this deletion has already been associated with GH-secreting adenomas phenotype [372 - 373], gastric carcinoid tumors and hyperparathyroidism [374].

	VARIANT	POSITION	NAME	ALLELES
1	rs34330	5'UTR	-79 T/C	T>C, *Fw:* T>C *Rev:* A>G
2	rs774454456	5'UTR	-26 delAGAG	*Fw:* -AGAG *Rev:* -TCTC
3	rs2066862	Exon 1	326 T/G V109G	T>G, *Fw:* T>G *Rev:* A>C
4	rs73330	3'UTR	*956 C/A	C>A, *Fw:* C>A *Rev:* G>T

Table 19: CDKN1B genetic variation generality

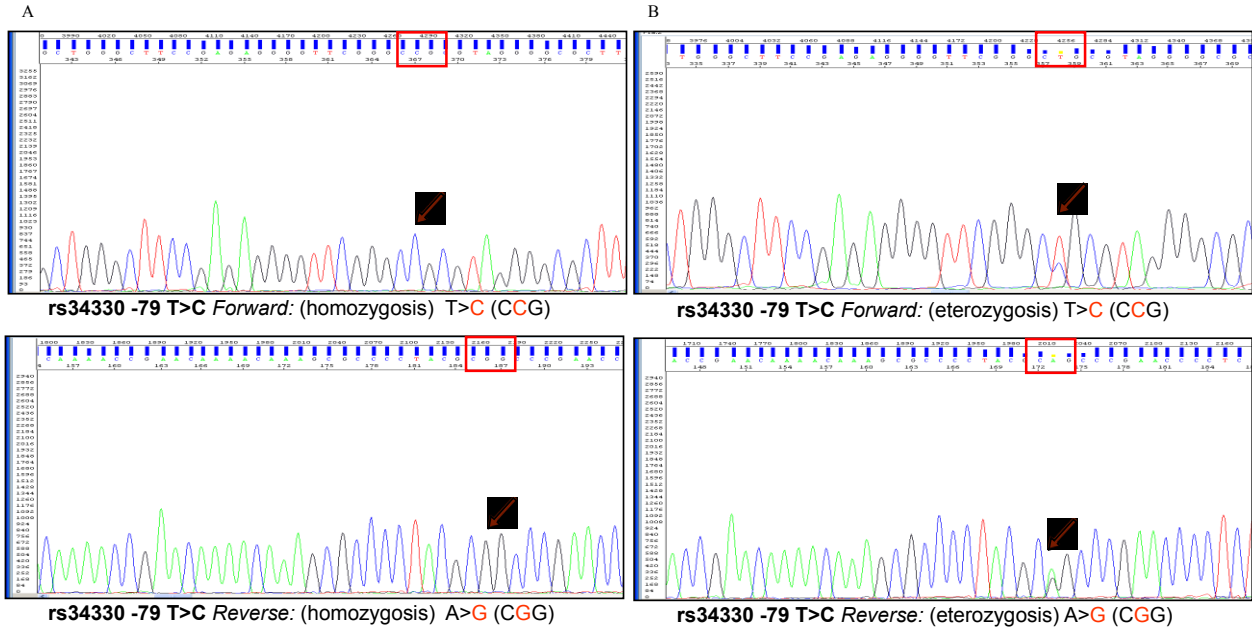


Figure 22: SNP electropherograms of -79 T>C (rs34330) in homozygosis (A) and heterozygosis (B).

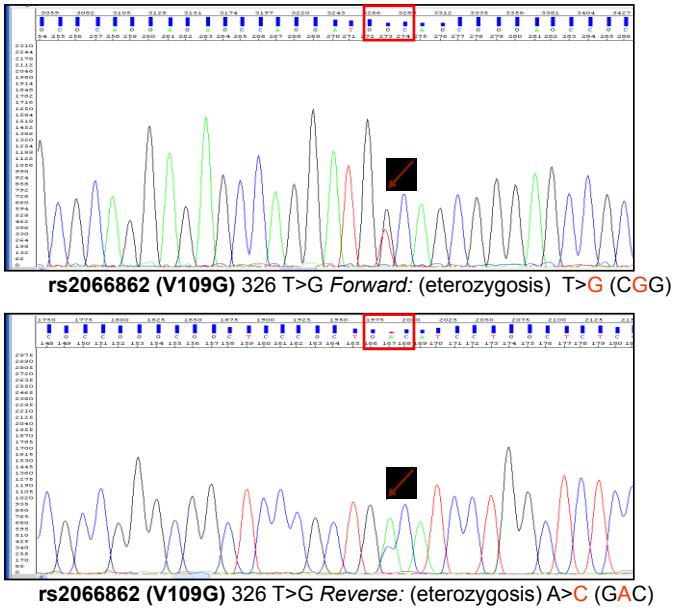


Figure 23: SNP electropherograms of 326 T>G (rs2066863, V109G) in heterozygosis.

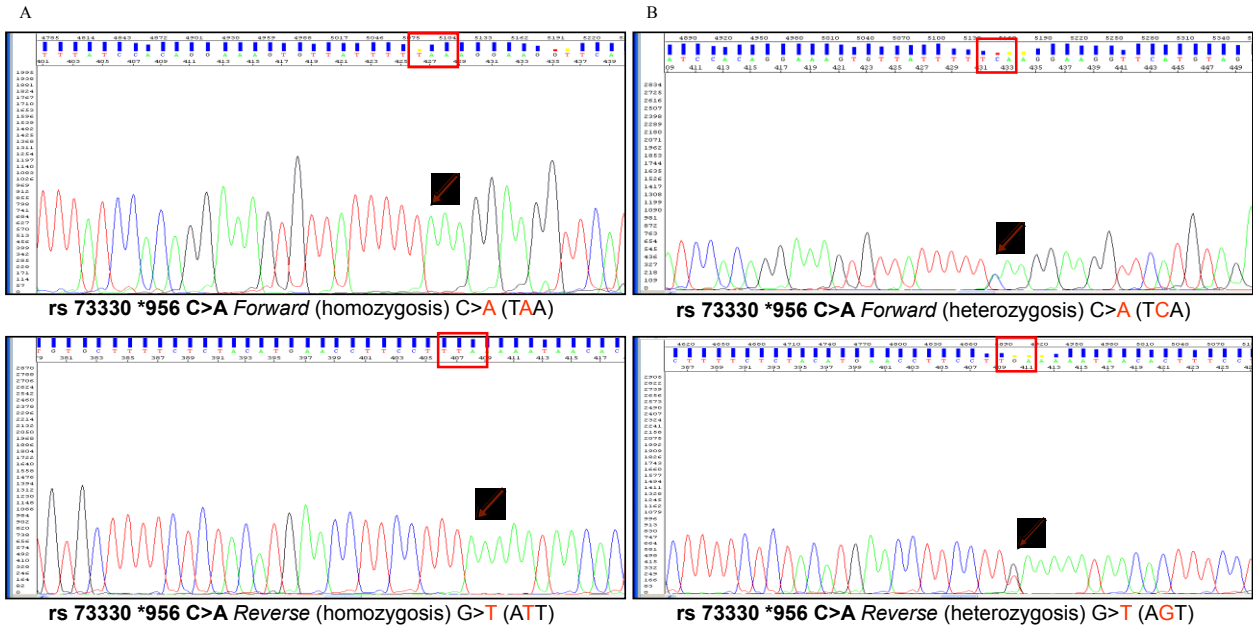


Figure 24: SNP electropherograms of *956 C>A (rs73330) in homozygosis (A) and heterozygosis (B).

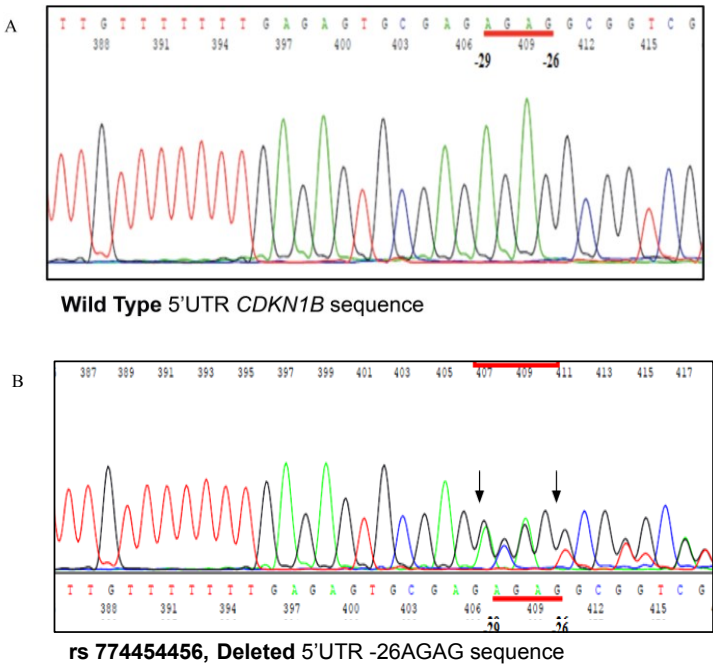


Figure 25: Wild Type (A) and deleted (B) electropherograms sequences (Deletion -26AGAG, rs774454456)

- *NCI-H727 and NCI-H720 BCs direct sequencing of CDKN1B gene:*

Direct DNA sequencing has shown the presence of three genetic variations, rs34330 -79 T/C (in homozygosis), rs2066862 V109G (in heterozygosis) and rs73330 *956 C/A (in homozygosis), in BCs NCI-H720 *Sensitive* to mTOR inhibitors treatment, while, in the BCs NCI-H727, *Resistant* to mTOR inhibitors treatment, only two of them genetic variations, rs34330 -79 T/C (in heterozygosis) and rs73330 *956 C/A (in heterozygosis) have been found.

- *Somatic direct sequencing of CDKN1B gene of 22 Human BC tissues DNA:*

Among the BC tissues samples patients 20/22 (90.9 %, 10 *Resistant* and 10 *Sensitive* to mTOR inhibitors) patients have shown the presence of rs34330 5'UTR -79 T/C base changing (8 in heterozygosis and 14 in homozygosis); only 2/20 (9.09%, both *Sensitive* to mTOR inhibitors) patients have shown the presence of rs774454456 5'UTR -26AGAG deletion; 6/22 (27.3%, 4 *Resistant* and 4 *Sensitive* to mTOR inhibitors) patients have shown rs2066862 exon1 V109G chancing (all in heterozygosis) and finally 18/22 (81.8%, 8 *Resistant* and 10 *Sensitive* to mTOR inhibitors) patients have shown the presence of rs73330 3'UTR *956 C/A base changing (9 in heterozygosis and 9 in homozygosis).

- *Germ-line direct sequencing of CDKN1B gene of 22 Human BC tissues DNA:*

14 peripheral blood samples, derived from some of the same patients analyzed for the somatic variations (paz. #1, 2, 3, 4, 6, 7, 9, 11, 13, 14, 15, 16, 17 and 18) have been collected and sequenced for CDKN1B gene; all patients have shown the same genetic SNPs patterns founded in the somatic DNA derived from BC tissues as well as the two deletions in the 5'UTR region. Overall among the peripheral blood samples patients 14/14 (100%, 5 *Resistant* and 9 *Sensitive* to mTOR inhibitors) patients have shown the presence of rs34330 5'UTR -79 T/C base changing (6 in heterozygosis and 8 in homozygosis); the same 2/20 (9.09%, both *Sensitive* to mTOR inhibitors) patients have shown the presence of rs774454456 5'UTR -26AGAG deletion; 4/14 (28.6%, 1 *Resistant* and 3 *Sensitive* to mTOR inhibitors) patients have shown rs2066862 exon1 V109G chancing (all in heterozygosis) and finally 14/14 (100%, 5 *Resistant* and 9 *Sensitive* to mTOR inhibitors) patients have shown the presence of rs73330 3'UTR *956 C/A base changing (7 in heterozygosis and 7 in homozygosis).

Subsequently a statistical analysis has been performed, on both cell lines and human BC sequencing data results, in order to understand firstly if it might be a relationship between the

genetic variants founded and the responsiveness to mTOR inhibitors observed, secondly if other variables, (like age, sex, diameter, Ki67, N metastasis and histology), could be related to the E-response. Frequency analysis (Box 4) distribution, assessed in human BC population sequencing data, have shown difference in the 5' UTR region (Ex1pI), as regard SNP rs34330 -79 T/C and rs774454456 -26AGAG, while, no differences have been found in the remaining gene regions (Exon1, Exon2, Exon 3 and boundaries indicated as Ex1pII-III, Ex2 and EX3a-b respectively).

EX1pI	Freq.	Percent	Cum.
None	2	9.09	9.09
Etero	8	36.36	45.45
Omo	10	45.45	90.91
Deletion	2	9.09	100.00
Total	22	100.00	

EX1pII	Freq.	Percent	Cum.
None	16	72.73	72.73
Etero	6	27.27	100.00
Total	22	100.00	

EX1pIII	Freq.	Percent	Cum.
0	22	100.00	100.00
Total	22	100.00	

EX2	Freq.	Percent	Cum.
None	22	100.00	100.00
Total	22	100.00	

EX3pA	Freq.	Percent	Cum.
None	4	18.18	18.18
Etero	9	40.91	59.09
Omo	9	40.91	100.00
Total	22	100.00	

EX3pB	Freq.	Percent	Cum.
None	22	100.00	100.00
Total	22	100.00	

Box 4: Frequency analysis genetic variation among the CDKN1B gene regions.

Comparing quantitative and qualitative variables in E-responder data patients versus E-resistant ones, no significant differences have been observed between responsiveness to mTOR inhibitors and sex, age and histology; nevertheless atypical histology and older people has mainly primary coltures resistant to E-treatment. Moreover, despite among the all population no significant differences have been found in the T staging, N staging, diameter, ki67 index and responsiveness to E, in older people bigger tumors, higher ki67 index and higher lymphonodal disease have been found. Whereas, exploring quantitative and qualitative variables in responder versus resistant to mTOR inhibitors treatment, and genetic variants detected, in 5'UTR region no significant differences have been found related to SNP rs34330 -79 T/C, although all deletions rs774454456 -26AGAG were detected in E-responder patients; as regard Exon1 rs2066862 V109G SNP, no significant differences, among the all population, have been found even if it seems that heterozygous is principally present in responder patients; Noteworthy is the relationship found between 3'UTR SNP rs73330 *956 C/A and responsiveness to mTOR inhibitors which significantly occurs, in homozygosis, in patients responsive to E-treatment (significance and statistic analysis data are shown in Box 5.

Key

frequency
expected frequency
column percentage

Enumerating sample-space combinations:
stage 3: enumerations = 1
stage 2: enumerations = 2
stage 1: enumerations = 0

ResponseRAD	EX3pA None	Etero	Omo	Total
Responder	1	4	6	11
	2.0	4.5	4.5	11.0
	25.00	44.44	66.67	50.00
Non Responder	3	5	3	11
	2.0	4.5	4.5	11.0
	75.00	55.56	33.33	50.00
Total	4	9	9	22
	4.0	9.0	9.0	22.0
	100.00	100.00	100.00	100.00

Pearson chi2(2) = 2.1111 Pr = 0.348
likelihood-ratio chi2(2) = 2.1772 Pr = 0.337
Fisher's exact = 0.550

Logistic regression — Number of obs = 22; LR chi2(2) = 2.18; Prob > chi2 = 0.3367; Log likelihood = -14.160622; Pseudo R2 = 0.0714

RADresponse	Odds Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
EX3pA						
Etero	.4166667	.5564229	-0.66	0.512	.0304146	5.70816
Omo	.1666667	.2256677	-1.32	0.186	.0117305	2.367997
_cons	3	3.464102	0.95	0.341	.3120602	28.84059

Box 5: 3'UTR SNP rs73330 *956 C/A and responsiveness to mTOR inhibitors significance and statistic analysis among human BC population.

Finally, according to BC cell lines NCI-H720 and NCI-H727 responsiveness to mTOR inhibitors and data regarding the genetic variations found in CDKN1B gene, it seems that a pattern of mutation similar to NCI-H720 predict response to E-treatment while NCI-H727 pattern mutation for a non response to E-treatment, (significance and statistic analysis data are shown in Box 6).

Key

frequency
expected frequency
column percentage

Enumerating sample-space combinations:
stage 3: enumerations = 1
stage 2: enumerations = 0
stage 1: enumerations = 0

ResponseRAD	Pattern Comparative to Cell Line No patter	NCI-H727	NCI-H720	Total
Responder	4	4	3	11
	4.0	4.5	2.5	11.0
	50.00	44.44	60.00	50.00
Non Responder	4	5	2	11
	4.0	4.5	2.5	11.0
	50.00	55.56	40.00	50.00
Total	8	9	5	22
	8.0	9.0	5.0	22.0
	100.00	100.00	100.00	100.00

Pearson chi2(2) = 0.3111 Pr = 0.856
likelihood-ratio chi2(2) = 0.3127 Pr = 0.855
Fisher's exact = 1.000

Logistic regression — Number of obs = 22; LR chi2(2) = 0.31; Prob > chi2 = 0.8553; Log likelihood = -15.09289; Pseudo R2 = 0.0103

RADresponse	Odds Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
PatternCellLine						
NCI-H727 (Typical)	1.25	1.218349	0.23	0.819	.1850382	8.444203
NCI-H720 (Atypi..)	.6666667	.7698004	-0.35	0.725	.0693467	6.40902
_cons	1	.7071068	0.00	1.000	.2500977	3.998438

Box 6: CDKN1B genetic variations and responsiveness to mTOR inhibitors significance and statistic analysis

10.9 IHC ON HUMAN BC SAMPLES WITH 5'UTR DELETION

In order to evaluate whether the concurrent presence of both CDKN1B rs34330 -79 T/C SNP and rs774454456 -26AGAG deletion could affect p27kip1 subcellular localization an IHC staining against p27kip1 protein has been performed on a BC patient sample that showed the presence of both gene variations (Figure 26)

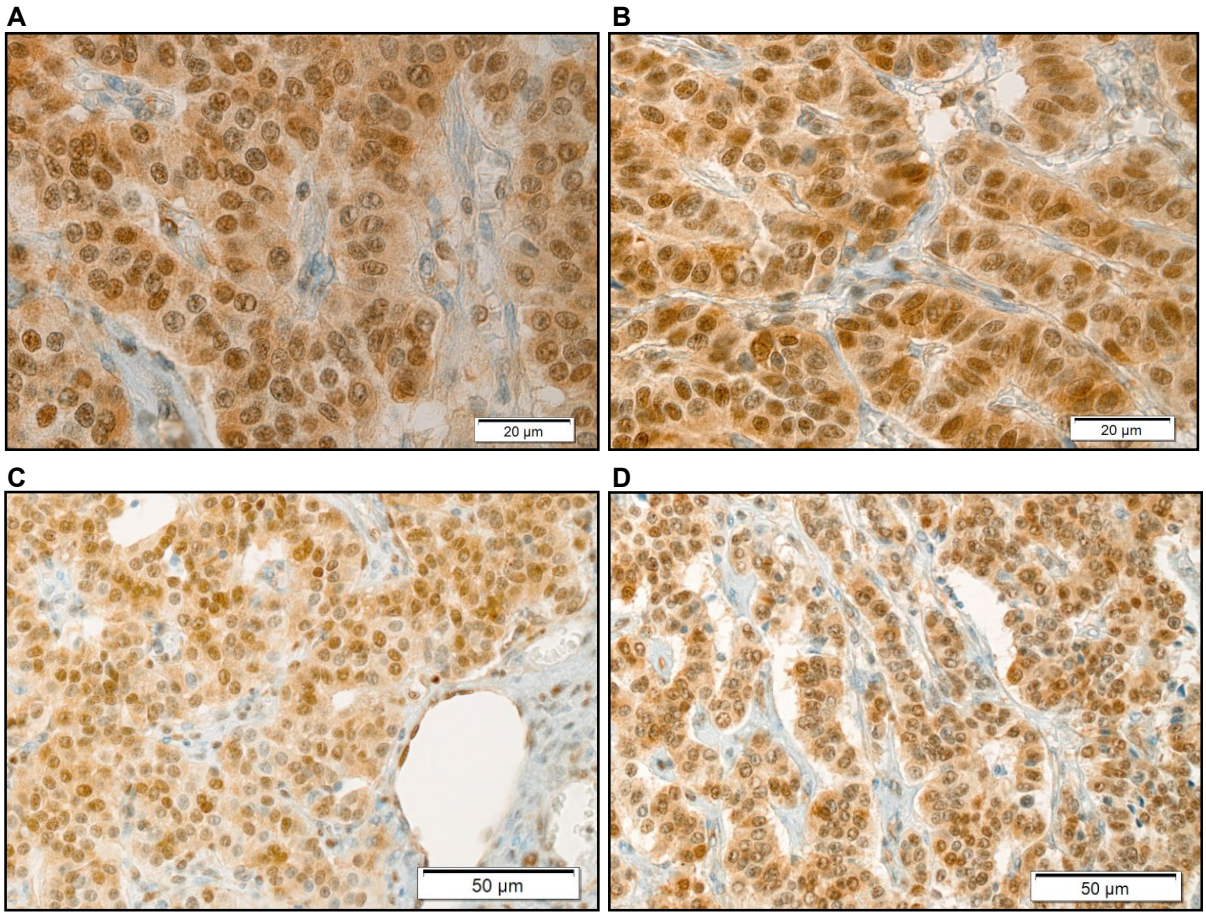


Figure 26: Immunohistochemistry for p27kip1 assessed on a human BC sample that showed the presence of both SNP **rs34330** -79 T/C and deletion **rs774454456** -26AGAG in 5'UTR CDKN1B gene.

As reported in *Fig. 26*, IHC analysis shows how $p27^{kip1}$ is manly located in the cytoplasm rather than in the nucleus, suggesting that the presence of these gene variations could affect its function and abundance. In effect, it is well known that, based on cellular localization, $p27^{kip1}$ plays its inhibitory function and/or may be trigged to ubiquitin-proteasome degradation mechanisms. To exert its Cdk inhibitory function, p27kip needs to be translocate to the nucleus [375], while increasing in nuclear export, through binding to CRM1 [294], is mainly related to kinases

degradation through phosphorylation-dependent mechanisms. Several recent reports have shown that cytoplasmic $p27^{kip1}$ has cell-cycle-independent functions, such as the regulation of cell migration that may be oncogenic under certain circumstances [376]; Moreover, it has been also previously reported [293] that $p27^{kip1}$ export may serves to remove inhibitory function from its nuclear targets (such as Cyclin E/Cdk2), rather than to promote degradation, which in turn may also impact on the activity of Cyclin D-Cdk complexes [377-378].

10.10 IDENDIFICATION OF DINACICLIB (SCH727965) EFFICAY RANGE CONCENTRATIONS

In general, 30% of human BC primary tissues shown a resistance to Everolimus (E) treatments [273]; based on our finding we suppose an association between deranged cell cycle Cyclin/Cdk protein overexpression, probably due to an impaired $p27^{kip1}$ function, and the resistance mechanisms observed in human BC responsiveness; Thus, we have tried to find out new putative medical treatment (i.e. Cdk Inhibitor Dinaciclib, SCH727965), which may be useful for those patients that do not respond to mTOR inhibitors, in order to overcame the resistance previously observed. Dinaciclib (D) is a novel promising Cdk Inhibitor, which has already shown encouraging, results in preclinical testing, in different leukemias types [379-380-381] and breast cancer [351-382]. In order to identify an efficacy range concentrations of D, we have assessed a concentration-scale treatment, (2 μM, 1 μM, 0,5 μM, 0,25 μM, 0,125 μM, 0,0625 μM, 0,03 μM, 0,0125 μM, 0,00625 μM), on both BCs, (Figure 27 and Figure 28), evaluating the responsiveness, in terms of cell viability variation and caspase 3/7 activation, after 48h and 72h of treatment. As shown in *Fig. 26,* D doesn't affect significantly cell viability in BCs sensitive to E, NCI-H720. After 48h, although cell viability is not affected by treatment with D, apoptosis activation has been observed, in particular in the range concentration comprised between 125 nM and 2 μM; this probably is linked to cell viability reduction observed after 72h of treatment, between 125 nM and 250 nM D treatments, which has however resulted non significant as compared to untreated control cells. Overall, D is not effective in E-sensitive NCI-H720 BCs, as this cell type hasn't shown a Cyclins/Cdks and linked $p27^{kip1}$ basal overexpression protein levels. On the contrary, D is effective in reducing cell viability and in activating apoptosis in BCs, NCI-H727, that display resistance to mTOR inhibitors, with related Cyclins/Cdks basal overexpression likely do to an impaired $p27^{kip1}$ function. As shown in *Fig. 27* after 48h, cell viability is significantly reduced by D treatment, until ~40% reduction rate, as compared to untreated

control cells, at almost all tested concentrations exception for 6 nM (after 48h and 72h treatments) and 12 nM (after 48h treatment); moreover, the viability reduction is enhanced after 72h of treatments, reaching ~60% reduction rate. Parallel to cell viability reduction a caspase 3/7 activation has been also observed, after 48h only at 12 nM and 30 nM of D, while, after 72h from 12 nM to 2 μM D treatments. Based on these finding two D concentrations have been chosen, 50 nM and 100 nM, for evaluating the effect of combined treatments with 100 nM E on both in vitro BCs and human primary BC cultures treatments.

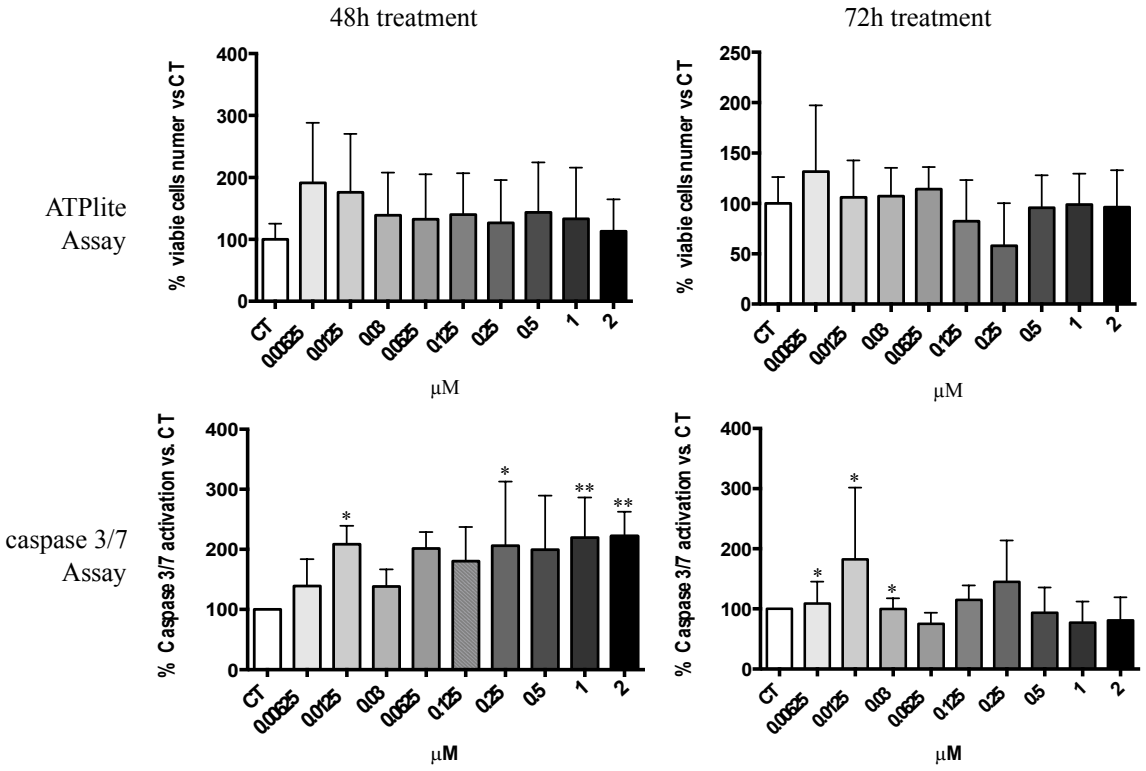


Figure 27: NCI-H720 BCs ATPlite Assays and Caspase 3/7 activation Assays regarding the concentration-scale (2 μM, 1 μM, 0,5 μM, 0,25 μM, 0,125 μM, 0,0625 μM, 0,03 μM, 0,0125 μM, 0,00625 μM) Dinaciclib treatments assessed after 48h and 72h of incubation time.

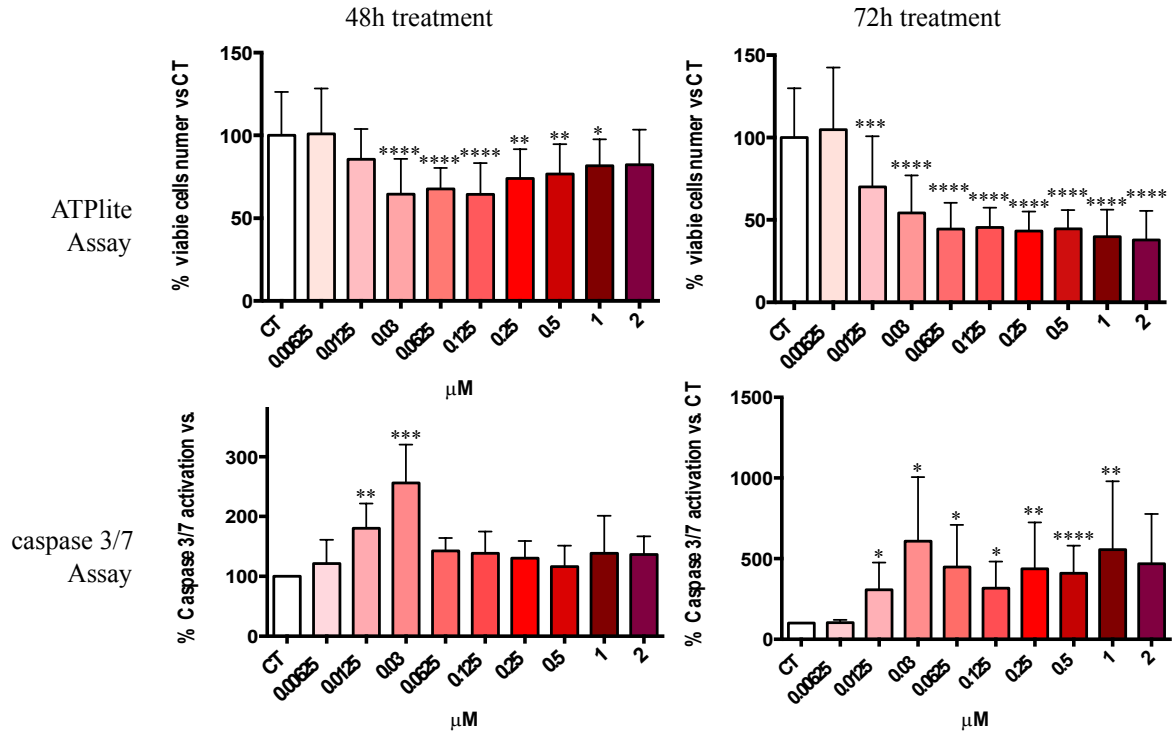


Figure 28: NCI-H727 BCs ATPlite Assays and Caspase 3/7 activation Assays regarding the concentration-scale (2 µM, 1 µM, 0,5 µM, 0,25 µM, 0,125 µM, 0,0625 µM, 0,03 µM, 0,0125 µM, 0,00625 µM) Dinaciclib treatments assessed after 48h and 72h of incubation time.

A		ATPlite Assays concentration-scale Dinaciclib columns statistic									
A1		NCI-H720									
	µM	CT	0.00625	0.0125	0.03	0.0625	0.125	0.25	0.5	1	2
48h	*Mean*	100	191,1	176,2	138,9	132,4	139,9	126,8	143,8	133,2	113,2
	Std. Deviation	25,44	96,89	94,14	69,02	72,85	66,97	69,01	80,76	82,71	51,36
	SEM	7,344	27,97	27,17	19,92	21,03	19,33	19,92	23,31	23,87	14,83
	ANOVA (Kruskal-Wallis test)	ns P value = 0,1815									
	Dunn's (multiple comparisons test)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
72h	*Mean*	131,5	106,2	107,2	114,3	82,29	57,9	95,56	98,86	96,2	
	Std. Deviation	65,75	36,56	28,07	21,94	40,97	42,25	32,47	30,83	36,67	
	SEM	15,5	8,618	6,617	5,171	9,656	9,959	7,652	7,267	8,643	
	ANOVA (Kruskal-Wallis test)	P value = 0,0002 (***)									
	Dunn's (multiple comparisons test)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
A2		NCI-H727									
	µM	CT	0.00625	0.0125	0.03	0.0625	0.125	0.25	0.5	1	2
48h	*Mean*	100	101	85,58	64,51	67,75	64,41	74,05	76,73	81,64	82,26
	Std. Deviation	26,3	27,35	18,35	21,35	12,53	18,92	17,6	18	15,99	21,23
	SEM	6,2	6,447	4,324	5,032	2,954	4,46	4,148	4,242	3,77	5,003
	ANOVA One Way summary	P value < 0,0001 (****)									
	Dunnett's (multiple comparisons test)	/	ns	ns	P < 0,0001	P < 0,0001	P < 0,0001	P < 0,005	P < 0,005	P < 0,05	ns
72h	*Mean*	100	104,8	69,95	54,23	44,48	45,47	43,2	44,6	39,82	37,84
	Std. Deviation	29,97	37,73	30,84	22,82	15,89	12	11,96	11,38	16,4	17,63
	SEM	7,063	8,893	7,269	5,379	3,744	2,828	2,818	2,682	3,866	4,155
	ANOVA One Way summary	P value < 0,0001 (****)									
	Dunnett's (multiple comparisons test)	/	ns	P < 0,005	P < 0,0001	P < 0,0001	P < 0,0001	P < 0,0001	P < 0,0001	P < 0,0001	P < 0,0001

Table 20: ATPlite Assays concentration-scale Dinaciclib columns statistic assessed on both NCI-H720 (A1) and NCI-H727 (A2) after 48h and 72h of treatments.

B		Caspase 3/7activation Assays concentration-scale Dinaciclib columns statistic									
B1		NCI-H720									
	µM	CT	0.00625	0.0125	0.03	0.0625	0.125	0.25	0.5	1	2
48h	*Mean*	100	139	208,6	138,6	201,4	180,7	206,1	199,6	219,7	221,9
	Std. Deviation	45,44	103,4	143,6	89,93	76,04	89,02	111	72,95	242,3	199,1
	SEM	13,7	31,19	43,3	27,11	22,93	26,84	33,47	22	73,06	60,03
	ANOVA (Kruskal-Wallis test)	P value = 0,0002 (***)									
	Dunn's (multiple comparisons test)	ns	P < 0,05	ns	ns	ns	P < 0,05	ns	P < 0,005	P < 0,005	ns
72h	*Mean*	100	108,9	182,5	99,97	75,02	114,8	145	93,61	77,15	80,87
	Std. Deviation	22,69	47,29	40,19	46,9	42,31	45,39	42,65	41,6	37,04	47,13
	SEM	5,673	11,82	10,05	11,72	10,58	11,35	10,66	10,4	9,259	11,78
	ANOVA One Way summary	P value < 0,0001 (****)									
	Dunnett's (multiple comparisons test)	ns	P < 0,05	P < 0,05	P < 0,05	ns	ns	ns	ns	ns	ns
B2		NCI-H727									
	µM	CT	0.00625	0.0125	0.03	0.0625	0.125	0.25	0.5	1	2
48h	*Mean*	100	121,4	180,3	256,2	142,5	138,4	130,4	116,4	138,8	136,4
	Std. Deviation	29,46	20,62	48,8	45,07	32,74	33,02	23,04	34,47	49,77	44,79
	SEM	6,944	4,861	11,5	10,62	7,717	7,782	5,431	8,126	11,73	10,56
	ANOVA (Kruskal-Wallis test)	P value < 0,0001 (****)									
	Dunn's (multiple comparisons test)	/	ns	P < 0,005	P < 0,005	ns	ns	ns	ns	ns	ns
72h	*Mean*	100	103,1	305,6	607,5	446,8	315,8	436,6	409,3	554,8	467,2
	Std. Deviation	21,36	53,68	76,85	62,23	79,07	69,83	81,47	134,1	73,71	28,69
	SEM	5,036	12,65	18,11	14,67	18,64	16,46	19,2	31,6	17,37	6,761
	ANOVA (Kruskal-Wallis test)	P value < 0,0001 (****)									
	Dunn's (multiple comparisons test)	/	ns	P < 0,05	P < 0,05	P < 0,05	P < 0,05	P < 0,005	P < 0,0001	P < 0,005	ns

Table 21: Caspase 3/7 activation Assays concentration-scale Dinaciclib columns statistic assessed on both NCI-H720 (B1) and NCI-H727 (B2) after 48h and 72h of treatments.

10.11 COMBINED EFFECT OF EROLIMUS AND DINACICLIB ON BCs NCI-H720 AND NCI-H727

In order to further understand the role of Cyclins/Cdks protein levels expression in mediating BC responsiveness to anticancer treatment in general, and moreover in the mTOR inhibitors resistance, combined treatment of D and E have been performed evaluating cell viability (Figure 29) and apoptosis processes (Figure 30) after 48h and 72h of incubation time, on both NCI-H720 and NCI-H727 BCs. Relative column statistic analysis are reported in Table 22 and Table 23.

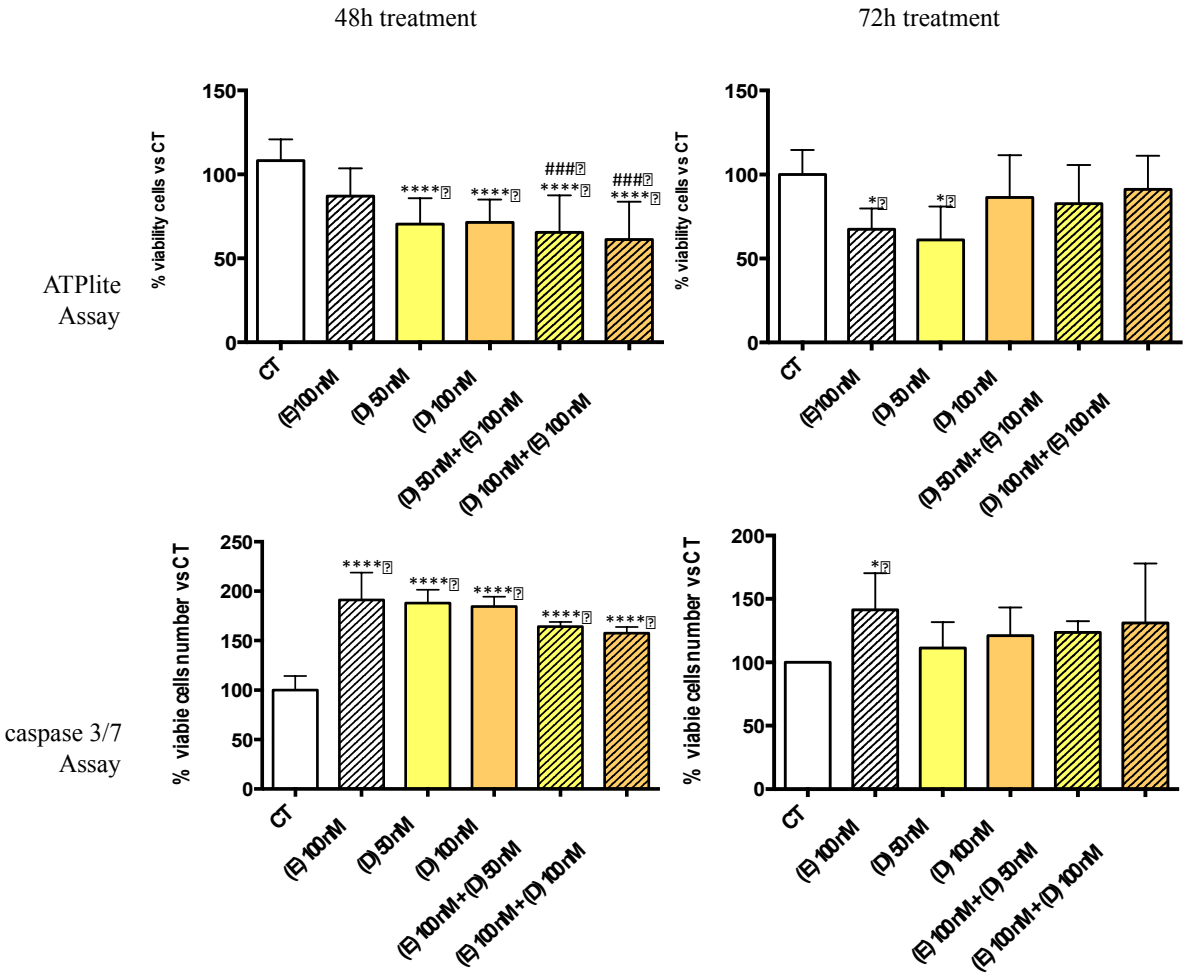


Figure 29: NCI-H720 BCs ATPlite and Caspase 3/7 activation Assays regarding the combined Everolimus - Dinaciclib treatments assessed after 48h and 72h of incubation time.

As attended and as already demonstrated [209-273], BCs NCI-H720 are sensitive to mTOR inhibitors treatment in terms of cell viability reduction and caspase 3/7 activation; as shown in *Fig. 28* treatment with E affect cell viability already after 48h of treatment (~13% rate of inhibition) which is also enhanced after 72h of incubation time (~33% rate of inhibition). Even thought treatment with D, as a single agent, significantly affect cell viability, with parallel apoptosis activation (~90% rate values of induction as compared to CT cells), after 48h of treatment, these cell lines are not sensitive anymore to D treatment after 72h of incubation time, exception for D 50 nM which affect cell viability but doesn't induce apoptosis activation, in correspondence to what we have seen in concentration-scale Assays and probably due to the absence od Cyclins/Cdks overexpression protein levels previously described (paragraph 9.1). Noteworthy is the slightly enhancing of cell viability reduction, but not in caspase 3/7 activation, observed after 48h of combined treatment (E 100 nM + D 100 nM), which is however loosed after 72h.

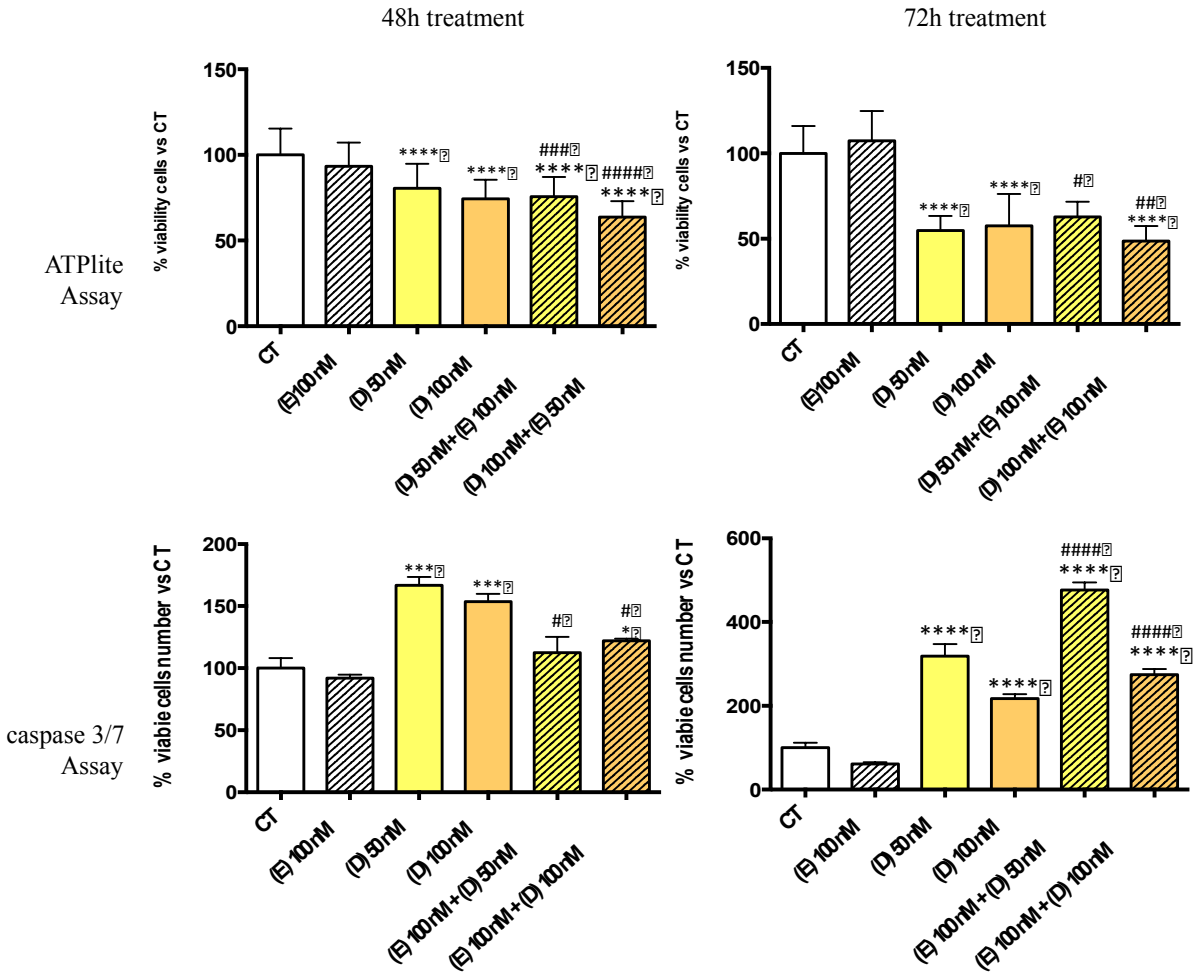


Figure 30: Figure 31: NCI-H727 BCs ATPlite and Caspase 3/7 activation Assays regarding the combined Everolimus - Dinaciclib treatments assessed after 48h and 72h of incubation time.

Combined treatment Assays-result, assessed on NCI-H727 BCs, are shown in *Fig. 29*; as already known these BCs is representative of E-resistant *in vitro* model and effectively treatment with E do not affect both cell viability and caspase 3/7 responses after both 48h and 72h of treatment. In agreement to what we have seen in concentration-scale Assays, D treatment, as a single agent, significantly affect cell viability, which, shows a ~20-25% rate values of inhibition as compared to untreated control cells, with a parallel caspase 3/7 activation as well (~40-50% more as compared to CT cells), after 48h of treatment; this inhibition, as well as apoptosis activation, are enhanced after 72h of incubation with the substance of interest, with a ~40-50% rate values in cell viability reduction, and a range rate values of apoptosis increasing comprised between ~200% and 400% rate values, as compared to untreated control cells. Moreover, the combination of D with E could overcome the resistance previously shown in NCI-H727 cells, since a slightly potentiation in cell viability reducing effects has been observed both after 48h and 72h of combined treatment (D 100 nM + E 100 nM combined treatment), as compared to single treatments (E 100nM, D 50 nM and D 100 nM).

A		ATPlite Assays combined treatment columns statistic					
A1		NCI-H720					
	nM	CT	E 100	D 50	D 100	R 100 + D 50	R100 + D100
48h	*Mean*	100	87,03	70,33	71,46	65,44	61,24
	Std. Deviation	7,855	16,59	15,44	13,53	22,1	22,41
	SEM	2,028	4,284	3,987	3,493	5,706	5,787
	ANOVA One Way summary	P vs. CT < 0,0001 (****), P vs. E = 0,0038 (##)					
	Dunnett's (multiple comparisons test vs. CT)	/	ns	P < 0,0001	P < 0,0001	P < 0,0001	P < 0,0001
	Dunnett's (multiple comparisons test vs E100)	/	/	ns	ns	P < 0,005	P < 0,005
72h	*Mean*	100	67,42	61,03	86,29	82,67	91,08
	Std. Deviation	14,61	12,35	19,81	25,17	22,95	20,05
	SEM	5,962	5,042	8,088	10,28	9,37	8,184
	ANOVA One Way summary	P vs. CT = 0,0240 (*), P vs. E ns = 0,0974 (##)					
	Dunnett's (multiple comparisons test vs. CT)	/	P < 0,05	P < 0,05	ns	ns	ns
	Dunnett's (multiple comparisons test vs E100)	/	/	ns	ns	ns	ns
A2		NCI-H727					
	nM	CT	R 100	D 50	D 100	R 100 + D 50	R100 + D100
48h	*Mean*	100	93,39	80,5	74,35	75,58	63,69
	Std. Deviation	15,46	13,86	14,38	11,17	11,45	9,385
	SEM	3,644	3,268	3,39	2,632	2,698	2,212
	ANOVA One Way summary	P vs. CT < 0,0001 (****), P vs. E < 0,0001 (####)					
	Dunnett's (multiple comparisons test vs. CT)	/	ns	P < 0,0001	P < 0,0001	P < 0,0001	P < 0,0001
	Dunnett's (multiple comparisons test vs E100)	/	/	P < 0,05	P < 0,0001	P < 0,005	P < 0,0001
72h	*Mean*	100	107,4	54,71	57,48	62,71	48,63
	Std. Deviation	16,02	17,36	8,567	18,76	8,927	8,765
	SEM	6,542	7,087	3,498	7,658	3,644	3,578
	ANOVA One Way summary	P vs. CT = 0,0002 (***), P vs. E = 0,0025 (##)					
	Dunnett's (multiple comparisons test vs. CT)	/	ns	P < 0,0001	P < 0,0001	P < 0,0001	P < 0,0001
	Dunnett's (multiple comparisons test vs E100)	/	/	P < 0,05	P < 0,05	P < 0,05	P < 0,05

E = Everolimus

D = Dinaciclib

P value vs. CT signed as (*) on the corressponding historgrams

P value vs. E 100 nM signed as (#) on the corresponding histograms

Table 22: ATPlite Assays combined (D) - (E) columns statistic, assessed on both NCI-H720 (A1) and NCI-H727 (A2) after 48h and 72h of treatments.

A	Caspase 3/7 Assays combined treatment columns statistic						
A1	NCI-H720						
	nM	CT	E 100	D 50	D 100	R 100 + D 50	R100 + D100
48h	*Mean*	100	191,0223	187,8528	184,4524	164,2363	157,5314
	Std. Deviation	18,8	47,88	30,03	20,3	11,6	12,58
	SEM	4,432	11,29	7,079	4,784	2,734	2,965
	ANOVA One Way summary	P vs. CT < 0,0001 (****), P vs. E = 0,0038 (##)					
	Dunnett's (multiple comparisons test vs. CT)	/	P < 0,0001	P < 0,0001	P < 0,0001	P < 0,005	P < 0,005
	Dunnett's (multiple comparisons test vs E100)	/	/	ns	ns	ns	ns
72h	*Mean*	100	141,4	111,3	121	123,7	131,2
	Std. Deviation	14,61	12,35	19,81	25,17	22,95	20,05
	SEM	5,962	5,042	8,088	10,28	9,37	8,184
	ANOVA One Way summary	P vs. CT = 0,0011 (**), P vs. E ns					
	Dunnett's (multiple comparisons test vs. CT)	/	P < 0,05	ns	ns	ns	ns
	Dunnett's (multiple comparisons test vs E100)	/	/	ns	ns	ns	ns
A2	NCI-H727						
	nM	CT	R 100	D 50	D 100	R 100 + D 50	R100 + D100
48h	*Mean*	100	91,86694	166,7536	153,6004	112,5239	122,0297
	Std. Deviation	18,19	6,503	15,03	14,03	28,08	3,929
	SEM	8,134	2,908	6,722	6,276	12,56	1,757
	ANOVA One Way summary	P vs. CT = 0,0027 (**), P vs. E = 0,0027 (##)					
	Dunnett's (multiple comparisons test vs. CT)	/	ns	P < 0,005	P < 0,005	ns	P < 0,05
	Dunnett's (multiple comparisons test vs E100)	/	/	ns	ns	P < 0,05	P < 0,05
72h	*Mean*	100	61,01118	318,6562	216,9303	476,6769	274,2126
	Std. Deviation	26,48	9,427	63,8	24,68	40,15	30,37
	SEM	10,81	3,849	26,05	10,08	16,39	12,4
	ANOVA (kruskal.Wallis test)	P vs. CT < 0,0001 (****), P vs. E < 0,0001 (####)					
	Dunn's (multiple comparisons test vs. CT)	/	ns	P < 0,0001	P < 0,0001	P < 0,0001	P < 0,0001
	Dunn's (multiple comparisons test vs E100)	/	/	P < 0,0001	P < 0,0001	P < 0,0001	P < 0,0001

E = Everolimus
D = Dinaciclib
P value vs. CT signed as (*) on the corressponding historgrams
P value vs. E 100 nM signed as (#) on the corresponding histograms

Table 23: Caspase 3/7 activation Assays combined (D) - (E) columns statistic, assessed on both NCI-H720 (B1) and NCI-H727 (B2) after 48h and 72h of treatments.

10.12 COMBINED EFFECT OF EVEROLIMUS AND DINACICLIB ON HUMAN BC PRIMARY COLTURES

In order to confirm the responsiveness to CDK-I Dinaciclib observed on *in vitro* BCs, human BC primary cultures, derived from patients' tumor tissues that underwent surgical resection for bronchial carcinoids (4 males and 4 females; age = 58.1 ± 5 years; median = 61; clinical characteristics are shown in Table 24), have been seeded and adequately treated with E (100 nM) and D (50 nM and/or 100 nM) as a single agents and as a combination of both; the responses

have been evaluated in terms of cell viability variation as shown in Table 25 (primary human BC coltures *Sensitive* to mTOR inhibitors) and Table 26 (primary human BC coltures *Resistant* to mTOR inhibitors).

No	SEX	AGE	SIDE	Ki67	TNM	Diameter (cm)	Histology
1	F	69	SX	1%	T1aN0Mx	1.2	Typical Carcinoid
2	M	77	SX	<1%	TaN0Mx	0,8	Carcinoid not sp.
3	F	38	DX	<1%	T2bN1Mx	5*7	Typical Carcinoid
4	F	69	DX	1%	T1N0Mx	1,7	Typical Carcinoid
5	F	57	SX	<1%	T2aN1Mx	3,5	Typical Carcinoid
6	M	43	DX	10%	T2aN2Mx	3	Atypical Carcinoid
7	M	65	SX	6%	T1BN1Mx	2,2	Atypical Carcinoid
8	M	47	DX	10%	T1aN0Mx	1,2	Atypical Carcinoid

Table 24: Patients' generality D – E combined treatment primary cultures.

Among the 8 primary coltures, 4 have shown a responsiveness to E-treatment, (grouped in Figure 32 and represented as green Box Plot), while 4 coltures have shown a resistance to E-treatment, (grouped in Figure 33 and represented as red Box Plot). Single treatments with D are reported as clear yellow Box Plots (D 50 nM) and/or clear orange Box Plots (D100 nM) while combined treatment with E 100 nM are shown as ticked orange and/or yellow Box Plots. In agreement to what we have seen in BC *in vitro* cell lines, D shows an efficacy on human BC primary coltures which resulted resistant to E-treatment (*Fig. 32,* tissues #3, 4 and 7); on the contrary, D is not effective in E-sensitive BC tissues and cell lines (*Fig. 31,* tissues #2, 6 and 8). Moreover, the combination of D with E, in some cases, can overcome the resistance previously against mTOR inhibitors treatment, as a more potent inhibitory effect has been observed in cell viability reduction of combined treatment with D and E as compared to single treatments. Based on these data we can conclude that D could represent a putative medical therapy for those patients showing resistance to mTOR inhibitors, which, in turn, may be linked to a deranged protein cell cycle profile.

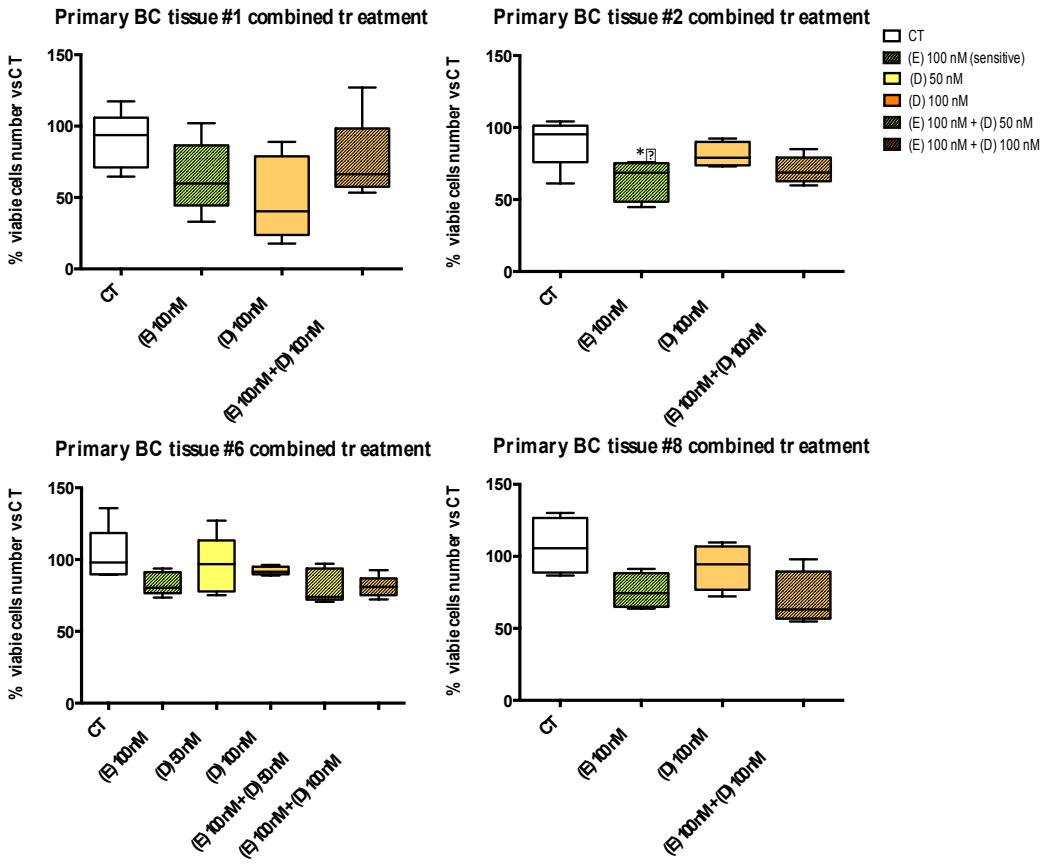


Figure 32: ATPlite Assays of single and/or combined treatmen D –E assessed on primary human BC coltures that have shown a responsiveness to mTOR inhibitors.

	Primary BC colture (#1)				Primary BC colture (#2)			
	CT	E 100 nM	D 100 nM	D 100 nM + E 100 nM	CT	E 100 nM	D 100 nM	D 100 nM + E 100 nM
Minimum	64,64	33,06	17,68	53,31	61,14	44,8	73,02	59,65
25% Percentile	71,12	44,4	23,75	57,35	75,99	48,52	73,76	62,62
Median	93,79	59,78	40,35	66,26	95,3	68,56	78,96	68,56
75% Percentile	105,9	86,5	78,81	98,25	101,2	75,25	90,1	78,96
Maximum	117,3	101,9	88,93	127	104,2	75,99	92,33	84,9
Std. Deviation	19,85	25,14	29,18	29,43	16,82	13,96	8,44	9,475
Std. Error of Mean	8,875	11,24	13,05	13,16	7,523	6,241	3,775	4,237
ANOVA (kruskal-wallis test vs CT)		P = 0,1528				P = 0,0465 (*)		
Dunn's test multiple comparison vs CT	/	ns	ns	ns	/	P < 0,05	ns	ns
ANOVA (kruskal-wallis test vs E 100nM)		P = 0,4585				P = 0,0783		
Dunn's test multiple comparison vs E 100 nM	/	/	ns	ns	/	/	ns	ns

P signicance P < 0,05 (*) or (#)

	Primary BC colture (#6)						Primary BC colture (#8)			
	CT	(E) 100 nM	(D) 50 nM	(D) 100 nM	(E) 100 nM + (D) 50 nM	(E) 100 nM + (D) 100 nM	CT	E 100 nM	D 100 nM	D 100 nM + E 100 nM
Minimum	89,48	73,57	75,2	88,95	70,64	72,3	86,64	63,65	72,14	54,79
25% Percentile	89,7	76,61	77,9	89,7	72,16	75,24	88,79	65,04	76,84	56,68
Median	98	80,41	96,88	91,55	73,95	80,96	105,6	74,41	94,49	63,12
75% Percentile	118,5	91,25	113,4	94,93	93,87	86,92	126,6	88,33	106,8	89,46
Maximum	135,7	93,79	127,1	96,26	97,1	92,65	130,2	91,23	109,7	97,98
Std. Deviation	19,05	7,995	20,33	2,851	11,86	7,405	19,76	12,17	15,73	19,24
Std. Error of Mean	8,521	3,575	9,091	1,275	5,305	3,312	9,879	6,083	7,867	9,618
ANOVA (kruskal-wallis test vs CT)				P = 0,0827					P = 0,0623	
Dunn's test multiple comparison vs CT	/	ns	ns	ns	ns	ns	/	ns	ns	ns
ANOVA (kruskal-wallis test vs E 100nM)				P = 0,0827					P = 0,0623	
Dunn's test multiple comparison vs E 100 nM	/	/	ns	ns	ns	ns	/	/	ns	ns

P signicance P < 0,05 (*) or (#)

Table 25: Colums statistic analysis referred to ATPlite Assays of single and/or combined treatmen D - E assessed on primary human BC coltures that have shown a response to mTOR inhibitors.

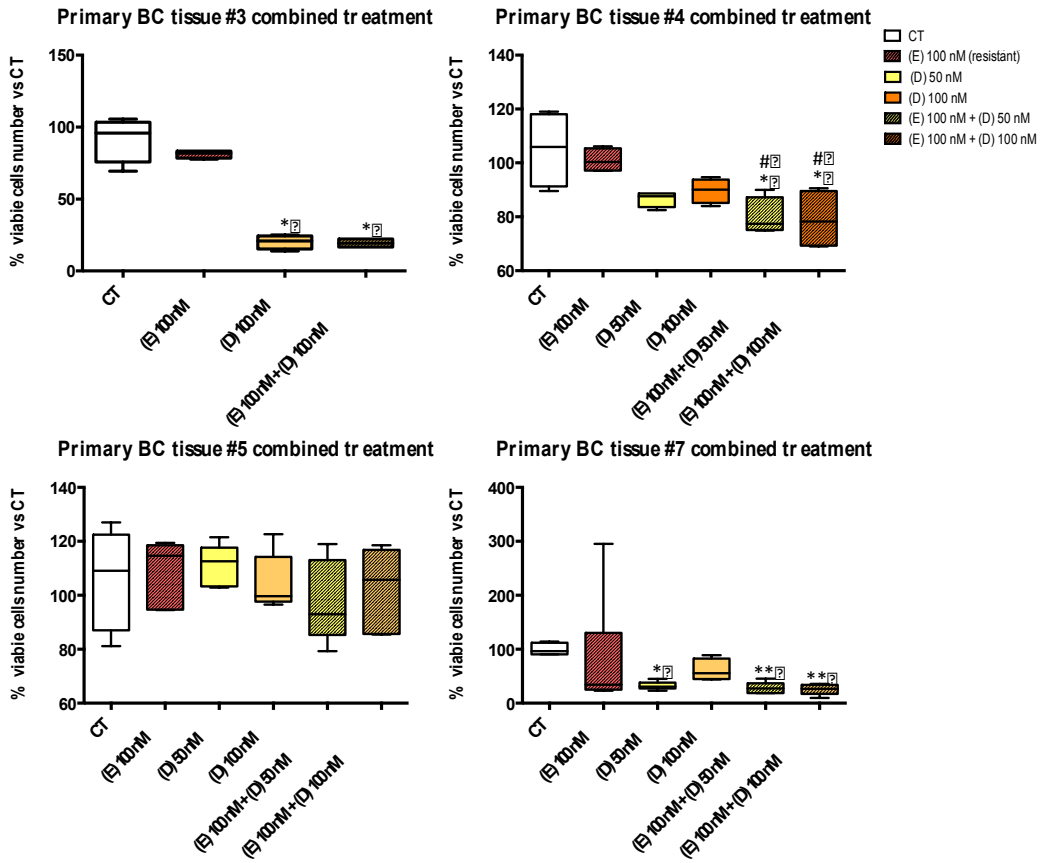


Figure 33: ATPlite Assays of single and/or combined treatment D –E assessed on primary human BC coltures that have shown a resistance to mTOR inhibitors.

	Primary BC colture (#5)						Primary BC colture (#7)					
	CT	(E) 100 nM	(D) 50 nM	(D) 100 nM	(E) 100 nM + (D) 50 nM	(E) 100 nM + (D) 100 nM	CT	(E) 100 nM	(D) 50 nM	(D) 100 nM	(E) 100 nM + (D) 50 nM	(E) 100 nM + (D) 100 nM
Minimum	81,15	94,56	102,8	96,54	79,32	85,48	90,32	23,32	23,14	43,93	18,59	9,843
25% Percentile	87,02	94,71	103,3	97,61	85,33	85,7	90,48	25,1	27,28	44,84	18,94	17,19
Median	109,1	114,6	112,6	99,69	93,03	105,8	96,72	34,46	30,7	55,72	27,13	26,57
75% Percentile	122,5	118,6	117,7	114,3	113	116,8	112,2	130,5	38,1	82,58	36,86	33,86
Maximum	127	119,4	121,5	122,6	119	118,5	114,2	295,2	45,21	89,09	45,43	35,87
Std. Deviation	18,6	12,47	7,736	10,62	15,32	15,73	10,41	106,4	7,504	19,31	10,2	9,472
Std. Error of Mean	8,316	5,576	3,46	4,749	6,852	7,037	4,249	43,44	3,063	7,884	4,163	3,867
ANOVA (kruskal-wallis test vs CT)				P = 0,07940						P = 0,0007 (***)		
Dunn's test multiple comparison vs CT	/	ns	ns	ns	ns	ns	/	ns	P < 0,05	ns	P < 0,005	P < 0,005
ANOVA (kruskal-wallis test vs E 100nM)				P = 0,06427						P = 0,0007 (###)		
Dunn's test multiple comparison vs E 100 nM	/	/	ns	ns	ns	ns	/	/	ns	ns	ns	ns

P signicance P < 0,05 (*) or (#)

	Primary BC colture (#3)				Primary BC colture (#4)					
	CT	E 100 nM	D 100 nM	D 100 nM + E 100 nM	CT	(E) 100 nM	(D) 50 nM	(D) 100 nM	(E) 100 nM + (D) 50 nM	(E) 100 nM + (D) 100 nM
Minimum	69,44	77,78	13,89	16,67	89,54	97,08	82,55	83,98	74,91	69,07
25% Percentile	75,69	78,48	15,28	16,67	91,3	97,19	83,59	85,2	75,17	69,28
Median	95,83	81,95	20,83	19,45	105,9	100,4	87,65	90,03	77,44	78,28
75% Percentile	103,5	83,33	24,31	22,22	118,1	105,4	88,64	93,79	87,23	89,6
Maximum	105,6	83,33	25	22,22	119	106,1	88,66	94,64	89,99	90,59
Std. Deviation	15,55	2,656	4,743	3,204	14,28	4,423	2,862	4,478	6,907	11,16
Std. Error of Mean	7,776	1,328	2,372	1,602	7,14	2,211	1,431	2,239	3,454	5,582
ANOVA (kruskal-wallis test vs CT)		P = 0,0082 (**)					P = 0,0074 (**)			
Dunn's test multiple comparison vs CT	/	ns	P < 0,05	P < 0,05	/	ns	ns	ns	P < 0,05	P < 0,05
ANOVA (kruskal-wallis test vs E 100nM)		P = 0,0119 (#)					P = 0,0162 (#)			
Dunn's test multiple comparison vs E 100 nM	/	/	P < 0,05	P < 0,05	/	/	ns	ns	P < 0,05	P < 0,05

P signicance P < 0,05 (*) or (#)

Table 26: ATPlite Assays of single and/or combined treatment D –E assessed on primary human BC coltures that have shown a resistance to mTOR inhibitors.

11. DISCUSISON AND CONCLUSIONS

Bronchial Carcinoid (BC) represents a heterogeneous group of NETs that arise from neuroendocrine cells widespread throughout the BP epithelium. They include both Typical and Atypical carcinoids and account for ~1-3% of all primary lung tumors, ~10% of all BP-NETs [117] and 20% of all well differentiated NETs [60-169]. The incidence of these neoplasms has increased significantly in recent decades (6% per year), due in part to early diagnosis imaging [168]. Traditional cytotoxic chemotherapy offers essentially minimal benefit to these largely under-characterized tumors [243] that still lack of medical therapy; to date, the mainstay and only curative treatment is represented by the complete surgical resection [177] of the primary tumor with the preservation of as much healthy lung parenchyma as possible. Due to the absence of specific symptomatology during the early stage of disease development, these neoplasms are often diagnosed accidentally when a lymphonodal and metastatic invasion is already displayed and surgery resection is not feasible; in this setting external radiotherapy is required and it is mainly used for palliation of bone or brain metastasis. Unlikely, BC have shown a resistance to radiation therapy [117] and medical therapy is required. The aims of medical treatment can be defined broadly as amelioration of symptoms and suppression of tumor growth and spread. Somatostatin analogues (SSA) remain the mainstay of symptomatic treatment for NETs [1]. Despite SSA represent the best therapeutic option for functional NETs they usually reduce hormone-related symptoms seldom showing an antitumor effects [185]; thus, it became evident the necessity to dispose of new medical treatment which might be effective in controlling the various featuring of this disease. Remarkable progress has been made over the last several years in knowledge of the molecular biology and treatment of NETs; the field has been transformed from one where patients have limited treatment options to one characterized by an increasing number of clinical trials and approved therapeutic agents. During about the last two decades, the better understanding of the mechanisms driving secretion and tumor growth has led to the development of several targeted antitumor agents in these rare tumors including anti-VEGF monoclonal antibodies, anti-EGF/IGFR and anti-PDGFR therapies and mTOR inhibitors, among the others. The PI3K/AKT/mTOR pathway deregulation is one of the most common mechanisms of tumorigenesis, which has been involved in the pathogenesis of familial and sporadic NETs [170]. In particular, mTOR has been identified as a kinase activated in the PI3K signaling pathway of lung NETs [239-240-242] in which, has been found constitutively activated. Its inhibition has shown antitumor effects, as a result of tumor cell growth inhibition [207], in several cancer types leading

to an increasing development of new therapeutic treatments during the last decade.

To date two rapamycin-derived mTOR inhibitors, Everolimus (E) and Temsirolimus, are used for NETs [244] management. Unfortunately, clinical updates indicate that rapamycin shows promise against only a few cancer types. Although E produced a significant prolongation of PFS in a number of patients with PNETs and lung carcinoids, they do not benefit from the drug owing to early or late progression, probably due to the development of primary or acquired resistance to E. Rapamycin inhibits mTOR activity but induces upstream signaling, leading to AKT activation, potentially limiting antitumor activity [264]. We have previously demonstrated [273] that approximately 70% of human BC primary cultures respond to E-treatment in terms of cell viability reduction paralleled with apoptosis activation; furthermore, in human BC cell line NCI-H720, *Sensitive* to mTOR inhibitors treatment, E induced a downregulation of Cyclin D1 protein levels parallel a higher cell accumulation in G_0/G_1 cell-cycle phase. On the contrary, in the *Resistant* BC cell line, the NCI-H727, the reduction in total Cyclin D1 protein levels does not correspond to a significant cell-cycle phase modification, suggesting that further mechanisms allow *Resistant* BC cells to bypass Cyclin D1 downregulation and cells continue to proliferate. The mechanisms underlying this phenomenon have not been fully clarified therefore the aim of our study was to further investigate the mechanisms involved in the resistance to mTOR inhibitors in human bronchial carcinoids, trying to find out new putative medical treatments. The progression through the cell cycle is governed by the periodic activation and inactivation of Cdk complexes, Ser/Thr protein kinases which activation is regulated by their association partners, called Cyclin [276]; Cyclin D1/Cdk4 together with Cyclin E/Cdk2 are positive regulators of G_0 to G_1/S cell-cycle transition. The Cdk activity is negatively controlled by association with Cdk inhibitors (CKIs); CKIs comprise the INK4 family and the Cip/Kip family which inactivate Cyclin/Cdk complexes and thereby cause growth arrest [278-284], one of Cip/kip member family is $p27^{Kip1}$, encoded by *CDKN1B* gene, which is a critical G_1/S cell cycle progression regulator. As BCs cell lines resistant to m-TOR inhibitors displayed a reduction in Cyclin D1 levels after treatment with E, despite they continue to proliferate [273], we investigated whether cell cycle protein profile of some of these regulators, included Cyclin E/Cdk2 and Cyclin D1/Cdk4, total $p27^{kip1}$ and phospoho SER10 $p27^{kip1}$, is different in the BC cell line sensitive to E as compared to the BC cell line resistant to E. We have observed that BC cell lines showed a different basal protein profile. Basal levels of Cyclin E/Cdk2, Cyclin D1/Cdk4, $p27^{kip1}$ and p-SER10 $p27^{kip1}$ are higher in the resistant BC cell line as compared to the sensitive one, suggesting a reduction in the inhibitory function of $p27^{kip1}$. Even thought in many human cancers loss or reduced $p27^{Kip1}$ protein expression is frequently observed [303], G_1 restriction-point disruption, in some tumors,

results in an unlimited growth through overexpression of certain checkpoint genes, such as Cyclin D, in parathyroid adenomas, or Cyclin E in breast cancer. In keeping with our findings, high $p27^{kip1}$ protein levels have been observed in many human cancers, such as an oesophageal cancer cell line [383], colon cancer samples [384], breast cancer [385], and small-cell carcinoma of the lung [386]. Furthermore, $p27^{kip1}$ over expression has been also described in tumours, associated with high Cyclin D/E protein levels. The latter finding characterises the proliferative drive of cancer cell, which, in turn, try to inhibit the hyper-proliferation status by inducing by $p27^{kip1}$ expression. However, this homeostatic mechanism is insufficient to hamper uncontrolled cancer cell proliferation [387]. After treatment with E, in BCs sensitive to m-TOR inhibitors treatments, despite both Cyclins D and E are reduced by treatment with E their Cdks are induced, suggesting that the G_0/G_1 cell cycle arrest E-mediated, is likely due to other mechanisms that excluded Cdks signal pathway (i.e. p21/p53). As attended total $p27^{kip1}$ is induced while phosphorylation on SER10 is reduced by treatments resulting in G_0/G_1 cells restrain. In BCs resistant to m-TOR inhibitors treatments, even though Cyclin D/Cdk4 and Cyclin E are inhibited by treatments with E, a plausible G_0/G_1 cell cycle arrest has not been observed, explaining the resistance showed by NCI-H727 BCs. We believe that this could be linked to the higher Cyclin D/E protein levels expression observed that lead to an impaired $p27^{kip1}$ function in NCI-H727 BCs which in turn try to inhibit the hyperproliferation by inducing $p27^{kip1}$ expression. In contrast, high $p27^{kip1}$ levels expression most likely results in a higher sensitivity to E-treatment giving the basis for the G_0/G_1 arrest escape and the consequent resistance to E. Our data indicate that resistance to mTOR inhibitors may be linked to a deranged cell cycle control protein profile likely due to an impaired $p27^{kip1}$ function. Our thought is also strengthened by the opposing $p27^{kip1}$/p-SER10 $p27^{kip1}$ protein levels trends observed among the BCs, before and after treatments with E. In particular, NCI-H720 BCs *Sensitive* to mTOR inhibitors, has shown a reduction of $p27^{kip1}$ phosphorylation at SER10, with an higher storage of total $p27^{kip1}$ as a consequence, explaining than the greater G_0/G_1 restrain previously observed after treatments with 100 nM of E [273]. As is common knowledge, $p27^{kip1}$ is phosphorylated on many sites; among the others, the phosphorylation of SER10 residue is regulated in a cell cycle-dependent manner and may function to stabilize $p27^{Kip1}$. It has been already reported that the extent of phosphorylation at this site is increased in resting cells [388] resulting in cytoplasmic export of $p27^{kip1}$ protein. Several recent reports have shown that cytoplasmic $p27^{kip1}$ has cell-cycle-independent functions, such as the regulation of cell migration that may be oncogenic under certain circumstances [376]; moreover, it has been also previously reported [293] that $p27^{kip1}$ export may serves to remove inhibitory function from its nuclear targets (such as Cyclin E/Cdk2), rather

than to promote degradation, which in turn may also impact on the activity of Cyclin D-Cdks complexes [377-378]. In keeping with these findings, in our results, E-*Resistant* NCI-H727 BCs, have shown higher basal p-SER10 $p27^{kip1}$ protein levels, as compared to NCI-H720 BCs, that, together with high Cyclins/Cdks and total $p27^{kip1}$ protein levels, confirm the higher proliferative trends of NCI-H727 BC cancer cells. Furthermore, as opposing to what it has been observed in E-*Sensitive* BCs, in NCI-H727 BCs treatments with Everolimus extremely affected total $p27^{kip1}$ levels, which results highly inhibited after E-treatments, leading to NCI-H727 G_0/G_1 escape likely due to loss of $p27^{kip1}$ cell cycle inhibition.

Subsequently, in order to confirm the putative role of $p27^{kip1}$ in mediating E-resistance mechanisms observed in BCs, total $p27^{kip1}$ and p-SER10 $p27^{kip1}$ protein levels expression have been evaluated in 22 human BC primary tissues. First of all, in order to establish the responsiveness to E, for each BC tissues patient's, primary coltures have been assessed and the response has been measured in terms of cell viability reduction and caspase 3/7 activation after treatment with E. In this way we defined as *Sensitive* those primary cultures displaying a significant reduction in cell viability ($P < 0.05$) and a significant increase in caspase activation ($P < 0.05$) after treatment with Everolimus. On the contrary, we defined as *Resistant*; those primary cultures, which cell viability and caspase activation, were not significantly affected by E-treatment. On this basis, we identified as *Sensitive* 11 BC primary cultures and as *Resistant* 11 BC primary. Than proteins were extracted from a portion of each BC primary tissue and a Western blot analysis for $p27^{kip1}$ and p-SER10 $p27^{kip1}$ was assessed; human BC tissues *Sensitive* to E treatment displayed higher total $p27^{kip1}$ and lower $p27^{kip1}$ p-SER10 protein levels as compared to *Resistant* BC tissues resulting in $p27^{kip1}$ index a significantly lower $p27^{kip1}$ index in human BC E-*Sensitive* tissues as compared to resistant ones. In keeping with findings obtained in BC *in vitro* cell lines, these data indicate that nevertheless $p27^{kip1}$ is expressed at high levels in sensitive BC tissues, it is less phosphorylated at SER10 as compared to human BC tissues resistant to treatments with mTOR inhibitors, confirming a putative role of $p27^{kip1}$ in responsiveness to mTOR inhibitors in human BC. $p27^{kip1}$ levels have been related to sensitivity of sarcoma cell lines to an antitumor macrocyclin called Aplidin [389] and they have also been positively correlated with the efficacy of a dual PI3K/mTOR inibitor, NVP-BEZ 235, as an antitumor agent [390]. In addition, higher $p27^{kip1}$ levels were recently found to be associated with improved response of breast cancer cell lines to temsirolimus and everolimus [391]. Although the use of $p27^{kip1}$ as a prognostic biomarker has long been established, its importance in the response to therapy in human cancers has started only recently to be investigated. To sum up, the characterization of cell-cycle protein profile, mainly focused on $p27^{kip}$ protein levels, may

represent a putative marker of resistance to E in human bronchial carcinoids, possibly contributing to a better patient's selection for a specific therapeutic approach. It is well known that a major challenge for the development of cancer therapy is the identification of predictive biomarkers of efficacy. To date, there are no known predictive biomarkers for the efficacy or resistance in cancer of mTOR inhibitors. Therefore, there is an emergent need to identify predictive markers of response that may be useful to prospectively select patients bearing tumors, which may respond, and benefit from mTOR inhibition therapies. $p27^{kip1}$ has good chances to became a putative biomarker for E-responsiveness in human BC; to further investigate its relationship with BC resistance mechanisms, in a second part of our study, direct sequencing of *CDKN1B* gene have been performed, on somatic/genomic BC patient's DNA (previously characterized for E-responsiveness) as well as DNA derived from the two BC cell lines employed in our study (NCI-H727 and NCI-H720). Unlike a variety of other well-characterized tumor suppressors, $p27^{Kip1}$ gene variations, such as deletions or mutations, are rarely observed in human cancers [314]; even thought numerous clinical studies have attempted to identify mutations within *CDKN1B* gene in individuals with cancer, such mutations have proved to be extremely rare [314-315]. Instead, several single nucleotide polymorphisms (SNP) have been identified in the human *CDKN1B* gene, including three, which are potentially functional (-838C>A rs36228499, -79C>T rs34330 and 326T>G V109G/rs2066827) [316]. Overall, among the 22 BC patients screened, four different genetic germ-line and somatic *CDKN1B* gene variations have been reported, both in heterozygosis and homozygosis: 1. SNPs rs34330 -79 T/C; 2. SNPs rs2066862 V109G; 3. rs73330 *956 C/A; 4. A 4bp deletion rs77445456 -26AGAG. In the 90.9% of the patients (20/22) SNPs rs34330 has been reported, while, the 27.3% of the patients (6/22) have shown the presence of SNPs rs2066862; finally in the 81.8% (18/22) BC patients SNPs rs73330 has been reported. SNPs -79C>T (rs34330), and 326T>G (V109G, rs2066827) are potentially functional, as previously reported [316]; in effect, epidemiological studies have found a significant association between V109G (rs2066827) and prostate cancer, breast cancer and oral squamous cell carcinoma [317], while, there are some evidences regarding the -79 T>C (rs34330) and the insurgence of papillary thyroid carcinoma [317]. Noteworthy, in addition to rs34330, a four bp deletion (rs77445456), has been found in two patients. We have recently detected this deletion in an acromegaly patient [368] and it has been correlated with a reduction in $p27^{kip1}$ mRNA levels, due to an altered secondary structure, which induces a reduced *CDKN1B* 5'UTR region transcriptional activity in vitro [368]. Moreover, this deletion has already been associated with GH-secreting adenomas phenotype [392 - 393], gastric carcinoid tumors and hyperparathyroidism [394]. Remarkable, genetic pattern observed in somatic DNA, derived from

primary tumor tissue of each patient, has also been confirmed in germ-line DNA, extracted from peripheral lymphocytes of available patients. The same *CDKN1B* gene variants, detected in human BC patients, have also been found in BC *in vitro* cell lines; in particular, rs34330 -79 T/C, rs2066862 V109G and rs73330 *956 C/A have been reported in BCs NCI-H720 *Sensitive* to mTOR inhibitors treatment, while, in the BCs NCI-H727, *Resistant* to mTOR inhibitors treatment, only two of them genetic variations, rs34330 -79 T/C and rs73330 *956 C/A have been found. Subsequently, in order to understand, firstly if it might be a relationship between the genetic variants founded and the responsiveness to mTOR inhibitors observed and secondly if other variables, like age, sex, diameter, Ki67, N metastasis and histology, could be related to the E-response, a statistical analysis ad been performed. Among the others, noteworthy is the relationship found between 3'UTR SNP rs73330 *956 C/A and responsiveness to mTOR inhibitors which significantly occurs, in homozygosis, in patients responsive to E-treatment; furthermore, according to BC cell lines NCI-H720 and NCI-H727 responsiveness to mTOR inhibitors and data regarding the genetic variations found in CDKN1B gene, it seems that a pattern of mutation similar to NCI-H720 predict response to E-treatment while NCI-H727 pattern mutation for a non response to E-treatment. Early detection of patients that may benefit from rapalogs treatment is of paramount importance in order to select the better treatment and avoid ineffective and expensive treatments; to date any genetic variation has been correlated to mTOR responsiveness in human BC and generally in NETs. Our understanding proved the role of CKI $p27^{kip1}$ in BC responsiveness to mTOR inhibitors, demonstrating that specific pattern CDKN1B gene variations may be helpful in devising more focused and effective targeted therapeutic strategies for mutation-positive patients.

The final goal of our study was to find out a new putative therapeutic treatment that could overcome the resistance to mTOR inhibitors in human BC. To briefly sum up, Cyclins/Cdks overexpression previously observed, probably resulting as a homeostatic mechanisms by which NCI-H727 E-Resistant BC cell line try to overcome a hyprproliferating status which, in turn, lead to $p27^{kip1}$ overexpression. Cyclin/Cdk complexes are overactive in most cancers and their pharmacological inhibition thus causes cell cycle arrest [338] and induces apoptosis selectively in transformed cells. Therefore, the inhibition of Cdks [339-340] has recently emerged as an attractive strategy for the development of novel oncology therapeutics since the inhibition of multiple members of the Cdk family has been shown to induce inhibition of proliferation and apoptosis [338-341]. *Dinaciclib* (D) represents a recent success as regards Cdk-I; it exerts its effects through the inhibition of various Cdks, such as Cdk 1, 2, 5, and 9, inducing growth inhibition of human cancer cells [351-352] by promoting apoptosis signal transduction pathway [351]. We have evaluated

how D could affect viability and/or apoptosis mechanisms on both in vitro BCs and primary human BC coltures; in addition, combined treatment with E have been assessed in order to investigate if they might be helpful in overcoming resistance to mTOR inhibitors. We found that D is effective in reducing cell viability and in activating apoptosis in human BC primary cultures and cell lines that display resistance to mTOR inhibitors (NCI-H727) and Cyclin-Cdk over-expression. On the contrary, D is not effective in E-sensitive BC tissues and cell lines (NCI-H720). Moreover, the combination of D with E can overcome the resistance previously observed, both on *in vitro* BCs and human BC primary coltures, with a more potent effect as compared to single treatments. Based on these data we can conclude that D could represent a putative medical therapy for those patients showing resistance to mTOR inhibitors, which, in turn, may be linked to a deranged protein cell cycle profile. D is currently in phase III clinical trials for CCL, AML and ALL leukemias [355-356-357], and more recently, preclinical studies assessed on breast cancer cells, proved its efficacy in inducing apoptosis and tumor regression [351-358].

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