La borsa di dottorato è stata cofinanziata con risorse del
Programma Operativo Nazionale Ricerca e Innovazione 2014-2020 (CCI 2014IT16M2OP005),
Fondo Sociale Europeo, Azione I.1 "Dottorati Innovativi con caratterizzazione Industriale"

XXXVI PhD Course in Applied Biology and Experimental Medicine

DEPARTMENT OF CHEMICAL, BIOLOGICAL,

PHARMACEUTICAL AND ENVIRONMENTAL SCIENCES

UNIVERSITY OF MESSINA

Dr.ssa Rossella Basilotta

SSD BIO/14

In Vitro and *In Vivo* Evaluation of Signaling Pathways Underlying the Tumor Suppressive Effect of Benzyl Isothiocyanate in Anaplastic Thyroid Carcinoma

Supervisor:
Chiar.ma Prof.ssa Emanuela Esposito

Coordinator:
Chiar.ma Prof.ssa Emanuela Esposito

Anno accademico 2022/2023

ABSTRACT

Anaplastic thyroid carcinoma (ATC) is a rare and aggressive form of thyroid cancer. It is often considered one of the most aggressive cancers known, with a very poor prognosis. ATC is characterized by its rapid growth and invasion into surrounding tissues. It is often diagnosed at an advanced stage when it has already spread to nearby structures in the neck, making it challenging to treat.

Due to its rarity and aggressive nature, anaplastic thyroid cancer is a difficult condition to treat and requires an early diagnostic approach and timely intervention, therefore knowledge of the mechanisms underlying thyroid cancer progression and identification of new therapeutic targets represent important research challenges. Benzyl isothiocyanate (BITC) is a naturally occurring compound found in cruciferous vegetables that has shown promise for its potential anticancer properties.

The aim of this study was to evaluate the antitumor effect of BITC in thyroid cancer, highlighting signaling pathways involved in BITC mechanism of action, and identify new therapeutic strategies for the treatment of the most common endocrine cancer. The study examined how BITC interacts with specific signaling pathways, including mTOR (mammalian target of rapamycin) and MAPK (mitogen-activated protein kinase). These pathways are often dysregulated in cancer and can be targeted for therapeutic purposes. This work included *in vitro* and *in vivo* studies. The *in vitro* model was performed using different TC cell lines to study the effect of BITC on the modulation of autophagy, through direct interaction with mTOR and MAPK pathways, and to evaluate its ability to modulate apoptosis and reduce cell migration. To confirm the *in vitro* findings and better mimic the complex tumor microenvironment, an in *vivo* orthotopic model of ATC was used. This involved the in-situ inoculation of ATC cells in mice, followed by treatment with BITC. Histological analysis of the mouse thyroids was conducted to evaluate the effects of BITC on tumor growth and

progression and Western blot analysis was used to examine markers related to autophagy, apoptosis, and epithelial-mesenchymal transition (EMT). The study's results indicate that BITC, both *in vitro* and *in vivo*, has the potential to slow the progression of anaplastic thyroid cancer. This effect appears to be mediated through interactions with autophagy pathways, reduction in EMT, and the attenuation of tumor-related inflammation. In conclusion, this study provides valuable insights into the potential role of BITC as a therapeutic agent for anaplastic thyroid carcinoma. It highlights the importance of understanding the molecular mechanisms underlying cancer progression and identifies BITC as a compound worth further investigation for the development of new treatment strategies for this aggressive form of thyroid cancer.

1. INTRODUCTION

Thyroid carcinomas (TC) are the most frequent malignancy of the endocrine system and present a great variability in terms of molecular, cellular and clinical characteristics. Based on these parameters, TC are divided into differentiated carcinomas such as Papillary Thyroid Carcinoma (PTC) and Follicular Thyroid Carcinoma (FTC), characterized by a favorable prognosis, and undifferentiated carcinomas such as Anaplastic Thyroid Carcinoma (ATC), lacking valid therapeutic possibilities [1]. This type of thyroid cancer typically arises from the follicular cells of the thyroid gland. It can develop de novo or may arise from pre-existing well-differentiated thyroid cancers, such as papillary or follicular thyroid carcinomas [2]. ATC is a rare and highly aggressive form of thyroid cancer and accounts for only a small percentage of all thyroid cancer cases but is responsible for a disproportionate number of thyroid cancer-related deaths due to its aggressive nature and poor prognosis [3]. The incidence of this neoplasia is three-fold higher in women and in individuals aged 25 to 65 years [4]. Diagnosis usually involves a combination of imaging studies, such as ultrasound, CT or MRI scans, and a thyroid nodule biopsy, but because of its tendency to progress rapidly ATC is often diagnosed at an advanced stage making timely treatment difficult [5]. Conventional therapies, including surgery and radioactive iodine (RAI) treatment, often significantly prolong overall survival (OS) and progression-free survival (PFS) of patients with well-differentiated thyroid cancer (WDTC), while more invasive and metastatic anaplastic thyroid cancer often cannot be surgically removed and has poor response to local therapies. Anaplastic tumor cells are not responsive to radioiodine and most modalities of chemotherapy and radiotherapy [6]. While targeted therapies for ATC have evolved rapidly over the past decade, drug resistance is a major obstacle to improving the prognosis of patients with advanced thyroid cancer [7].

Although many efforts have been made to improve the accuracy in the characterization of these tumors, the understanding of the mechanisms that regulate the differentiation of tumor cells and

the identification of new therapeutic targets are important steps in the treatment of thyroid carcinomas. Most of the recently discovered targeted therapies for ATC inhibit the known oncogenic mechanisms in thyroid cancer initiation and progression such as MAPK pathway, PI3K/Akt-mTOR pathways, or VEGF [8]. Benzyl isothiocyanate (BITC), a bioactive natural product found in cruciferous vegetables, has shown anticancer effects through modulation of apoptosis, inflammation, and autophagic pathways. BITC has been shown to inhibit cancer progression in preclinical animal testing [9-11]. Some studies report the beneficial effect of BITC in the inhibition of both basal and Hepatocyte growth factor (HGF)-stimulated migration and in the invasion of breast cancer cells, effects that seem to be attributable to urokinase-type plasminogen activator (uPA) downregulation and Plasminogen activator inhibitor-1 (PAI-1) upregulation, which in turn may be mediated by suppression of Akt activation [9]. In addition, orthotopic xenograft models of breast cancer have documented the inhibition of EMT by BITC, through up-regulation of adherent junction proteins (E-cadherin and occludin) and down-regulation of mesenchymal markers such as vimentin and fibronectin and suppression of E-cadherin transcriptional repressors (snail and slug) [12]. Further works showed that BITC treatment inhibited the expression of pro-survival proteins IGF1R, mTOR and FGFR3 by upregulation of miR-99a in bladder cancer cell lines, providing strong preclinical evidence for miRNA-based treatment of cancer [13]. The most reported signaling pathway with which BITC interacts is certainly autophagy. BITC is known to affect the mammalian target of rapamycin (TOR) pathway, which is a key regulator of autophagy, potentially affecting the balance between cell survival and cell death in cancer cells. BITC's ability to induce autophagy in cancer cells has led to research exploring its potential therapeutic applications. Some studies suggest that combining BITC with autophagy inhibitors or other cancer therapies could improve its effectiveness in treating certain types of cancer [14,15]. Research on BITC and autophagy has been conducted in various types of cancer, including breast cancer, prostate cancer, colon cancer, and more [16-18]. The specific effects of BITC on autophagy may vary depending on

the type of cancer cell and context. BITC causes inhibition of cytoprotective autophagy of human gastric adenocarcinoma AGS cells by decreasing both autophagy initiator proteins and lysosomal degradation [10], while it has demonstrated antitumor activity in human prostate cancer cells through the induction of early protective autophagy pathways by inhibiting mTOR signaling regardless of their sensitivity to androgens [19]. However little attention has been paid to the impact that BITC could have on the progression of thyroid cancer. The aim of this work was to evaluate, through both *in vitro* and *in vivo* models, the antitumor effect of BITC in ATC, highlighting signaling pathways involved in its mechanism of action.

This research aiming to evaluate the impact of BITC on ATC represents a significant effort to address a challenging and deadly cancer type. Understanding how BITC influences ATC progression and identifying relevant signaling pathways can potentially lead to the development of innovative and more effective therapeutic approaches for ATC patients, offering hope for improved outcomes and quality of life.

CHAPTER TWO: THYROID CARCINOMAS

2. THYROID CARCINOMAS

2.1 Definition

Thyroid carcinoma (TC) is the most frequent neoplasm of the endocrine system, accounting for about 95% of all endocrine cancers and about 2.5% of all malignancies. TCs are characterized by great variability in terms of cellular, molecular, genetic, and clinical characteristics. This important phenotypic heterogeneity generally results from the integration of genetic and non-genetic influences and may have implications in understanding the mechanisms underlying therapeutic resistance and in the development of effective strategies. Recent advances in multiscale profiling of these tumors have improved understanding of the tumor landscape and led to the potential identification of novel biomarkers and molecular targets for prognosis and therapeutic intervention [20].

2.2 Etiopathogenesis

The etiological factors involved in the development of TCs include both constitutional predispositions and genetic etiological factors. Among the risk factors involved in the oncogenesis of the most studied TCs is certainly radiation exposure. In fact, numerous evidences have revealed the close correlation between radiation exposure (e.g. local irradiation for the treatment of infections or inflammation of the tonsillar and nasopharyngeal region or even acne and thymus irradiation therapy) and the significant increase in the risk of developing thyroid carcinomas [21]. The facts for the deleterious effect of radiation exposure in the development of thyroid cancer have been confirmed in the population of Hiroshima and Nagasaki following the accident at the Chernobyl nuclear power plant [22]. An increase in the incidence rate was detected in the first 3-4 years after the accident, due to exposure to radioisotope ^{131} I, especially in the younger population in the age group of 4 years. The assessed relative risk exists at an average exposure dose of 10 cGy and above this exposure

dose up to 1500 cGy there is a linear dependence between irradiation and risk of developing TCs. Higher exposures above 1500 cGy are associated with a relative risk reduction, probably due to the cytotoxic effect of these high doses of exposure [23].

Another environmental factor related to the etiopathogenesis of TCs is dietary iodine intake (nutritive iodine deficiency or sufficiency) [24]. Both low and high iodine intake conditions can result in changes in TSH and stimulate the development of carcinogens. According to some investigations, iodine deficiency compared to its excessive intake has an impact more like a promoter, not a direct carcinogen, causing the increase in TSH and the consequent stimulation of thyroid EGF (epidermal growth factor) and the reduction of TGFβ1 (transforming growth factor β1), with concomitant promotion of angiogenesis and tumor growth. To confirm this evidence, numerous studies have shown a high incidence of follicular and anaplastic carcinomas in the population coming from iodine-deficient regions [25]. Among the constitutional etiological factors related to the development of TCs there is pre-existing benign thyroid disease. According to several case-control studies and prospective studies, conditions such as benign nodular/multinodular thyroid disease, goiter, autoimmune disorders (Graves' disease and Hashimoto's disease), can lead to an increased risk of developing TCs [26]. In addition, there was a significant difference in the incidence rate of TCs between females and males after puberty and in the reproductive period [27]. This evidence therefore suggests the possibility that estrogenic effects are also related to the development of TCs. Another risk factor identified through numerous case-control studies is the condition of obesity. The exact pathophysiological mechanism is not fully understood, but the increase in TSH related to this condition could lead to an altered interaction with insulin-like growth factor 1, causing the inactivation of the MAPK and PI3K pathways that may be enrolled in pathogenesis of TCs. Recent developments in molecular biology have unveiled several genetic alterations associated with the oncogenesis of TCs. Key mutations include those in the RET gene (15-33% of cases), RAS mutations (10%), and BRAF mutations (40-

60%). Additional molecular alterations involve signaling pathways such as the MAPK and PI3K-AKT pathways. The MAPK pathway, crucial for tumor initiation, is activated in thyroid carcinomas through RET/PTC rearrangement [28], RAS mutations, and BRAF mutations [29]. This pathway consists of a network of three kinases that activate each other through sequential phosphorylation in response to various stimuli and it regulates cellular processes such as cell growth, differentiation, and adaptation to stress. In the RAF-RAS/MEK/ERK pathway, signal transduction begins with the activation of RAS through various membrane proteins. RAS mutations, found in follicular adenomas, lead to permanent activation. There are three RAS isoforms (HRAS, KRAS, NRAS) found in thyroid cancers, with mutations occurring early in premalignant lesions. The deletion of KRAS and concurrent deletion of PTEN may trigger malignant transformation into aggressive forms of follicular thyroid carcinoma (FTC). Mutations and deletions of the tumor suppressor gene PTEN activate the PI3K-AKT pathway are exceedingly rare in papillary thyroid carcinoma (PTC) but contribute to 80% of FTC and in 2–11% of Poorly differentiated thyroid carcinomas (PDTC) and in 12–39% of Anaplastic thyroid carcinoma (ATC) [30-32]. While RAS mutations are characteristic of follicular thyroid malignancies, RET/PTC proto-oncogenes are predominantly found in PTC. Similar to their well-differentiated counterparts, *BRAF* and *RAS* mutations remain the main drivers in PDTC and ATC, occurring in 33% and 45% of PDTC, and 29% and 23% of ATC respectively [33,34]. BRAF mutations, specifically the transverse point mutation T1799A resulting in BRAF-V600E expression, are detected in about 45% of PTCs, activating serine/threonine kinase [35]. The tumor suppressor gene TP53, located on chromosome 17, may undergo mutations leading to dedifferentiation in tumor evolution. Inactivating TP53 mutations, a genetic hallmark of ATC [36], are infrequent in well-differentiated thyroid carcinoma, being detected in merely 0.8% of PTC, but are highly prevalent in ATC, detected in up to 73% of tested ATC [32-34]. Genetic rearrangements, such as the PAX8/PPARγ rearrangement (t(2;3)(Q13;p25)), result in the creation of oncogenic proteins in follicular

variant FTC (13%) [37]. In addition, mutations in the TERT promoter appear to be associated with a more aggressive phenotype [38]. In fact, they are detected at low frequency in well-differentiated thyroid cancer: 10% of PTC, 17% of FTC, while on the contrary high mutation rates of the TERT promoter occur in 40% of PDTCs and 73% of ATCs, respectively [39]. Understanding these molecular processes has facilitated in-depth investigations into the molecular pathogenesis of thyroid carcinomas.

2.3 Classification

The fifth edition of the World Health Organization's (WHO) Classification of Endocrine and Neuroendocrine Tumors was released in March 2022 [40] and lists the different tumor types within a hierarchical taxonomic classification that is assigned based on the cell of origin, pathological or molecular characteristics, and biological behavior. The four main taxonomic ranks are category, family (class), type, and subtype. Most thyroid cancers arise from follicular epithelial cells, while a small number arise from calcitonin-secreting C cells. Follicular cell-derived malignancies are classified into benign tumors, low-risk malignancies, and malignant malignancies. The classification of thyroid tumors has evolved on the basis of classical histopathology and molecular pathogenesis.

2.3.1 Benign follicular cell-derived thyroid tumors

The category of benign thyroid neoplasms includes: follicular nodular disease (FND), follicular adenoma, follicular adenoma with papillary architecture and oncocytic follicular adenoma. Follicular nodular disease (FND) is a term proposed by the new edition of the WHO classification to indicate all conditions that were previously referred with the clinical term "goiter". In fact, this term refers to an enlargement of the thyroid gland associated with various neoplastic and non-neoplastic disorders. FDN therefore includes all pathologies previously

defined with the terms "colloidal nodules", "multinodular goiter", "adenomatous goiter" and "multinodular hyperplasia" often used by pathologists, but which did not reflect the underlying pathology beyond the mere confirmation of clinical findings. Follicular adenoma is a common benign neoplasm of the thyroid gland. It is an encapsulated tumor characterized by a solid or rubbery, homogeneous, round, or oval appearance surrounded by a thin fibrous capsule. Papillary follicular adenoma is an encapsulated, well-demarcated, non-invasive tumor characterized by intrafollicular centripetal papillary growth and lack of the nuclear features of papillary thyroid carcinoma (PTC) [41]. These tumors are often associated with autonomous hyperfunction and may therefore appear as hot or warm nodules on radionuclide thyroid scan [41]. Molecular analyses have shown that these are driven by TSHR, GNAS or EZH1 mutations and alterations that activate the protein kinase A (PKA) pathway [42,43]. Moreover, non-functional follicular adenomas with papillary architecture can harbor DICER1 mutations, and a subset of these have been reported in association with DICER1 syndrome [44-46]. Oncocytic thyroid tumors represent a distinct entity of thyroid malignancies, characterized by the presence of 75% of tumor cells with oncocytic features. This neoplasm is supported by specific genetic aberrations including mitochondrial DNA mutations and increased copy number alterations [46,47].

2.3.2 Low-risk follicular cell-derived thyroid neoplasms

The fourth edition of the WHO Classification of Thyroid Cancers introduced a group of malignancies termed "low-risk." This category encompasses non-invasive follicular neoplasm of the thyroid with papillary-like nuclear features (NIFTP), thyroid tumors of uncertain malignant potential, including follicular thyroid tumors of uncertain malignant potential (FT-UMP), well-differentiated tumors of uncertain malignant potential (WD-UMP), and trabecular meshwork hyalinizing tumors (HTT) [1]. When assessed using stringent diagnostic

criteria, these malignancies collectively exhibit clinically benign behavior and typically do not necessitate aggressive treatment modalities. It's important to note that HTT is an exception to this trend. Most of these low-risk malignancies are predominantly driven by RAS mutations. However, the identification of non-RAS-like molecular signatures (e.g., BRAF p.V600E) or high-risk molecular alterations (e.g., TERT promoter mutations) warrants a re-evaluation of pathological features to exclude overt malignancy. This underscores the significance of a comprehensive molecular and pathological assessment for accurate classification and determination of appropriate treatment strategies. NIFTP is a well-demarcated or encapsulated, non-invasive neoplasm characterized by follicular matrix with PTC-related nuclear atypia lacking high-grade features (mitosis/necrosis) [48,49]. Most NIFTPs are RAS-driven or have BRAF codon 601 (K601) and BRAF p mutations, therefore the V600E mutation represents an exclusion criterion for NIFTP. Thyroid tumors of uncertain malignant potential are rare types of neoplasm in which the histological confirmation of capsular and/or vascular invasion is unclear, so they require extensive microscopic evaluation, because the presence of a single invasive focus would exclude this type of diagnosis. HTTs are follicular cell-derived thyroid neoplasms with PTC-related nuclear atypia, trabecular growth pattern, extracellular hyaline matrix and specific PAX8::GLIS1 and PAX8::GLIS3 fusions [50].

2.3.3 Follicular thyroid carcinoma and follicular variant of papillary thyroid carcinoma

Follicular thyroid carcinomas are mostly RAS-driven invasive tumors that lack PTC nuclear cytology. FTCs are divided into three different subtypes, each reflecting the overall prognosis based on tumor capsule invasion and vascular invasion foci: minimally invasive FTC, encapsulated angioinvasive FTC, and extensively invasive FTC [1]. The related follicular variant of PTC (FVPTC) is similar to FTC in terms of predominant growth patterns but

exhibits thinner nuclear features than most PTCs that are linked to molecular alterations similar to BRAF p.V600E. FVPTCs are now divided into infiltrative and encapsulated subtypes. Infiltrative FVPTCs are BRAF-driven tumors with florid nuclear atypia with invasion of the surrounding thyroid parenchyma and lymphatic vessels (thus behaving like real PTCs). Encapsulated FVPTCs are RAS-driven lesions with an invasive pattern similar to FTCs (capsular and/or propensity for vascular rather than lymphatic invasion) [1]. Similar to FTC, FVPTCs also show a correlation between the extent of invasion and the patient's prognosis [51].

2.3.4 Papillary thyroid carcinoma

Papillary Thyroid Carcinoma (PTC) is the most common type of thyroid cancer, accounting for about 80% of all thyroid cancer cases. It is characterized by distinctive features, including papillary structures (finger-like projections) and nuclear abnormalities such as nuclear grooves and psammoma bodies (calcified structures). In recent years, much attention has been paid to the histological subtyping of PTCs [1]. PTCs driven by oncogenic BRAF mutations account for 50-60% of all cases and are specifically overrepresented in high-cell, columnar cell, and hobnail subtypes. This subtype correlates with the higher risk of lymph node metastases, loco-regional recurrences, distant metastases, and poor outcomes compared to RAS- driven PTC counterpart [1]. In addition to the classic PTC, several histological subtypes in terms of histological definitions and molecular profiles are now listed. The most common are infiltrative, high-cell, columnar cell, nail-cell, solid, diffuse sclerosing, Warthin-like and oncocytic PTCs.

2.3.5 Poorly differentiated thyroid carcinoma and differentiated high-grade thyroid carcinoma

Poorly differentiated thyroid cancer (PDTC) is a subset of differentiated thyroid carcinomas (PTC, FTC) that exhibit high-grade pathological features such as increased mitotic index and the presence of tumor necrosis and it is associated with an aggressive clinical course [52,53]. The diagnosis of PDTC is made on the basis of the so-called Turin criteria; (1) presence of a solid/trabecular/insular growth pattern, (2) absence of the conventional nuclear features of papillary carcinoma, and (3) presence of at least one of the following: convoluted nuclei; >3 mitosis per 2 mm2 (10 high-power fields) and tumor necrosis [54]. In the latest revision of the WHO classification, an intermediate entity of "high-grade differentiated thyroid cancer" (DHGTC) is therefore introduced for high-risk differentiated thyroid carcinomas that retain PTC-related nuclear atypia or a follicular growth pattern, but that exhibit features that are not acceptable for a diagnosis of PDTC, such as ≥ 5 mitoses per 2 mm^2 and/or tumor necrosis [1]. DHGTCs and PDTCs are lesions guided by BRAF or RAS, because they are tumors preceded by a PTC or FTC. Interestingly, DHGTCs are mostly derived from *BRAF*-driven PTCs, while PDTCs often exhibit aberrant *RAS* signaling, indicating a relationship between FTCs and FVPTCs [55].

2.3.6 Anaplastic thyroid carcinoma

Anaplastic thyroid carcinoma (ATC) is a highly aggressive thyroid tumor constituted of undifferentiated follicular cells. This rare type of thyroid carcinoma (1–2% of all thyroid malignancies) usually develops in elderly patients, presenting rapidly growing, firm and infiltrative neck mass. The prognosis of ATC is severe with a median survival period of less than 6 months and mortality rate of more than 90% [56]. Three histological types (sarcomatoid,

giant cell, and epithelial) are observed in any combination and proportion in ATC. Nuclear pleomorphism, tumor necrosis, increased mitosis, and infiltrative growth are key features for a conclusive diagnosis of ATC, and immunohistochemistry Ki67 is useful to confirm ATC and other high-risk thyroid carcinomas. In the previous WHO classification, squamous cell carcinoma (SCC) of the thyroid gland was listed as a separate entity from anaplastic thyroid carcinoma (ATC); however, studies have shown that both behave clinically similarly. Molecular profiling solved this diagnostic issue; the vast majority of thyroid SCCs harbor BRAF p.V600E mutations and show immune-expression of TTF1 and PAX8, demonstrating the origin from follicular cells [57] [58]. In addition, thyroid SCCs can be seen in combination with a well-differentiated thyroid cancer, and similarly, a subset of ATCs exhibits easily recognizable squamous differentiation, making it logical to classify thyroid SCCs as a morphological model/subtype of ATC rather than a separate entity. The new classification also encourages routine use of BRAF p.V600E mutation-specific VE1 immunohistochemistry in all patients with ATC, given the potential benefit of BRAF and MEK inhibitor therapies [1].

2.3.7 Medullary thyroid carcinoma

Medullary thyroid carcinomas (MTCs) constitute approximately 5–10% of all thyroid cancers. Although the tumor forms in the thyroid, it doesn't originate from thyroid cells, but from the C cells or parafollicular cells which produce and release a hormone called calcitonin (CT) [59]. MTC are defined as high-grade tumors based on the presence of at least one of the following parameters: tumor necrosis, mitotic count ≥5 per 2 mm^2, and/or a Ki67 proliferation index ≥ 5% [60] .

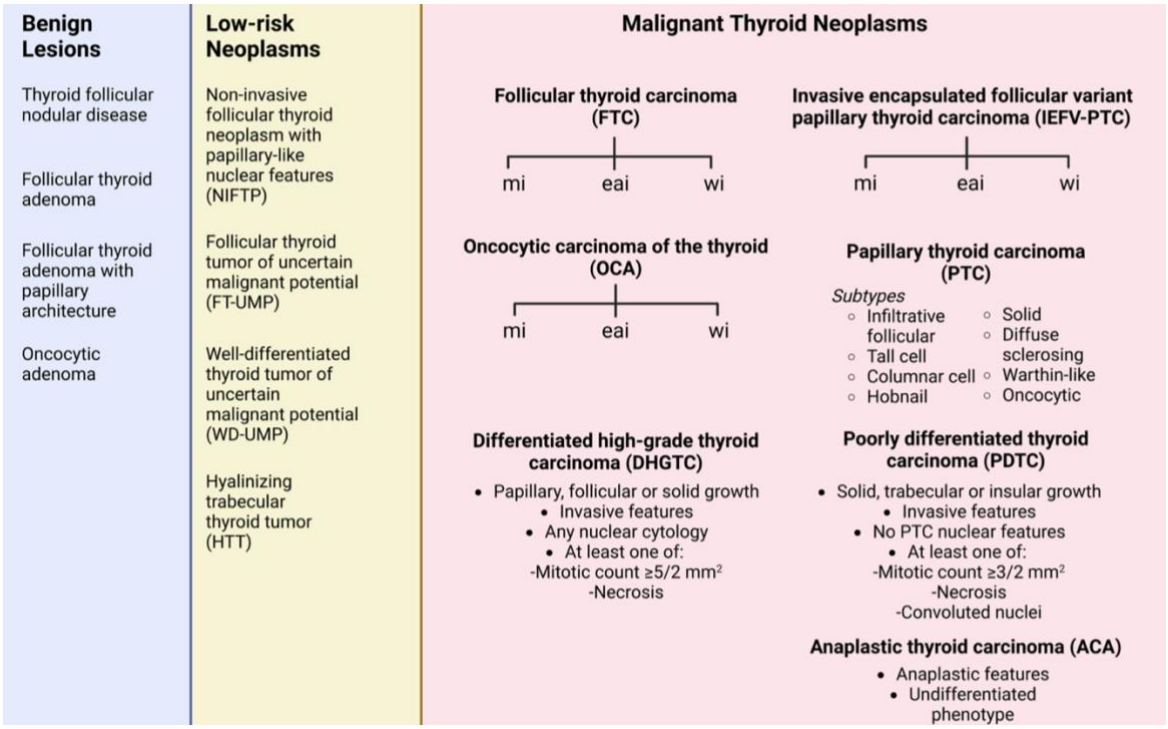

Figure 1. Overview of the main diagnostic groups of the 2022 WHO classification of thyroid tumors. MI, minimally invasive; EAI, encapsulated angioinvasive; WI, widely invasive. [Christofer Juhlin C, Mete O, Baloch ZW. The 2022 WHO classification of thyroid tumors: novel concepts in nomenclature and grading. Endocr Relat Cancer. 2022 Dec 22;30(2):e220293. doi: 10.1530/ERC-22-0293. PMID: 36445235].

2.4 Diagnosis

The diagnostic process of TCs (including poorly differentiated forms) includes pre- and postoperative pathological and molecular evaluations. Guidelines for the diagnosis of TCs recommend thyroid ultrasound along with examination of cervical lymph nodes in patients with suspected thyroid nodules. When thyroid ultrasound is performed, the clinical goal is to detect nodules at high risk of thyroid cancer. The presence of findings such as microcalcifications, irregular margins, and marked hypoechogenicity indicate a higher risk of malignancy. Existing guidelines classify thyroid nodules into risk categories based on the suspected features mentioned above and make recommendations for biopsy. In the Thyroid Imaging Reporting and

Data System (TI-RADS) developed by the American College of Radiolology (ACR) the nodules were then reclassified and evaluated in terms of echogenic foci, margin irregularity, taller-than-wide shape, and calcification and microcalcification. The risk category of the nodules was scored according to the following features. Nodules were classified as benign (TR1, 0 points), very low suspicion (TR2, 2 points), low suspicion (TR3, 3 points), intermediate suspicion (TR4, 4–6 points), and high suspicion (TR5, ≥7 points) [61]. According to the guidelines fine needle aspiration biopsy (FNAB) is recommended at 1 cm and above for high or moderate suspicious nodules, 1.5 cm and above for low-suspicion nodules, and 2 cm and above for ultra-low-suspicion nodules [62]. Cytological findings are classified into diagnostic categories associated with different risks of malignancy [63]. Most malignant thyroid tumors can be identified cytologically. Notable exceptions are FTCs and non-invasive follicular thyroid neoplasm with nuclear papillary-like features (NIFTP), which are usually classified as indeterminate in the various thyroid cytology reporting schemes [64]. FNAB-based diagnosis of poorly differentiated carcinoma is also difficult unless there is an obvious increase in mitotic activity and/or necrosis. The diagnosis of FNAB may be facilitated by the evaluation of markers of malignancy (including proteins commonly overexpressed in tumors, e.g., HBME1 or galectin-3) and molecular profiling to detect alterations specifically associated with malignancy (e.g., BRAF-, RAS-, *TERT*-, TP53-, RET- mutations, other new genetic alterations). Resected TCs are histologically classified according to World Health Organization (WHO) criteria (updated in 2022) and mentioned previously.

2.5 Epidemiology

The incidence of thyroid cancer has been steadily increasing since the 1980s and appears to be related to geographic location. In fact, it is found to be higher in higher-income countries, including the Republic of Korea, Canada, Italy, France, Israel, Croatia, Austria, and the United States, as well as in some upper-middle-income countries, such as Turkey, Brazil, Costa Rica,

and China. The incidence is also high in some island nations and territories, including Cyprus, Cape Verde, French Polynesia, New Caledonia and Puerto Rico. This variation is thought to be primarily attributable to geographical differences in access to care and diagnostic practices, although environmental exposures may also play a role [65]. Globally, in 2020, the age-standardized incidence rates of thyroid cancer were 10,1 per 100 000 women and 3,1 per 100 000 men. Compared to incidence, thyroid cancer death rates tend to be much lower and vary much less based on geographic location, in fact, in 2020 age-standardized mortality rates were 0,5 per 100 000 women and 0,3 per 100 000 men [66]. Thus, mortality has remained very low and stable over time compared to incidence. The rising incidence coincided with the introduction and increasingly widespread use since the 1980s-90s of medical imaging techniques, including thyroid ultrasonography, and sensitive diagnostic tools [67], resulting in the incidental detection and diagnosis of cancers that would previously not been detected. For these reasons, the rising incidence of thyroid cancer has been referred to as an "epidemic of overdiagnosis" [68]. Overdiagnosis is defined as the diagnosis of a condition that would not have caused harm to the individual over their lifetime if left undetected. Similar trends in incidence have been observed in nearly every region of the world, including some lower-resource countries, without clear corresponding increases in mortality. Thyroid cancer incidence increases from adolescence through middle age, peaking around 55 years in women and 65 years in men, and subsequently declining with older age. The prognosis for thyroid cancer is typically excellent, because most cases are PTCs are localized to the thyroid gland at diagnosis, in fact the 5-year relative survival is 98.6% overall, 99.9% for localized, 98.3% for regional, and 54.9% for distant metastatic disease [69].

2.6 Treatments

Clinical practice guidelines in oncology for thyroid cancer address the management of thyroid cancers depending on the type of tumor and are different for papillary, follicular, medullary, and anaplastic cancer. Thanks to innovations in recent decades, most patients can be cured of this disease if properly treated. The treatment of choice is surgery, followed by radioactive iodine (RAI) ablation (iodine-131) in selected patients and thyroxine therapy in most patients.

2.6.1 Surgery

Before performing surgery, it is necessary to evaluate the extent of thyroidectomy on the basis of the type of tumor, family history, and the coexistence of bilateral thyroid disease. Guidelines recommend total thyroidectomy for patients with biopsy-proven PTC under the following circumstances: T3 or higher, clinical N1 disease, M1 disease, aggressive subtype, significant radiation exposure, significant family history, or coexisting thyroid disease. While unilateral lobectomy is recommended as appropriate management for most patients with papillary and follicular carcinoma based on (1) the low mortality and low recurrence rates among most patients (those patients categorized as low risk); and (2) the high complication rates reported with more extensive thyroidectomy. Lobectomy (without RAI ablation) is recommended for PTC patients who have small volume incidental pathologic metastases (<5 lymph nodes involved without metastases >2 mm, in the largest dimension) [62]. For patients with FTC, who in most cases have benign disease, total thyroidectomy is recommended only if interoperative findings of extrathyroidal extension or lymph node metastases are evident at the time of surgery. Otherwise, lobectomy is advised as the initial surgery for follicular neoplasia on FNAB and it is preferred also for minimally invasive cancers, as well as NIFTP tumors, followed by surveillance, because usually they have an excellent prognosis. Instead, completion thyroidectomy is recommended for any of the following cases: tumor greater than 4 cm in diameter, positive resection margins, gross extrathyroidal extension, multifocal macroscopic

disease (>1 cm), macroscopic lymph node metastases, confirmed contralateral disease, or vascular invasion [62]. Surgery is also the main treatment for MTC. Total thyroidectomy and bilateral central neck dissection (level VI) are indicated in all patients with MTC whose tumor is ≥1 cm or who have bilateral thyroid disease; total thyroidectomy is recommended, and neck dissection may be considered for those whose tumor is <1 cm and for unilateral thyroid disease [70]. For patients diagnosed with ATC, the possibility of local resection should be evaluated [71]. If the disease involves the larynx, trachea, esophagus, pharynx, carotid artery, and other structures of the neck, surgery should be thoroughly evaluated by an experienced surgeon who can perform complex neck surgery. If the patient appears to have resectable disease, an attempt at total thyroidectomy with complete resection of the gross tumor, with resection of all involved local or regional structures and lymph nodes, should be performed, although this type of approach has not been shown to prolong survival. However, most patients with ATC have unresectable or metastatic disease [72].

2.6.2 Radioactive Iodine

Radioactive iodine (RAI), also known as radioactive iodine or iodine-131 (I-131), is a radioactive isotope of iodine, and it is often used as the primary treatment for DTC after thyroidectomy. It involves the administration of radioactive iodine to destroy any remaining thyroid tissue or cancer cells. RAI for removal of postsurgical gland remnants is typically not indicated for patients classified as having a low risk of disease-specific recurrence/mortality; adjuvant therapy with RAI may be considered for patients with intermediate-risk disease without gross residual disease, but data are conflicting regarding the benefit of RAI in this setting; RAI treatment is often used for patients with known postoperative residual disease or inoperable distant metastases based on whether the persistent tumor has been shown to be greedy for iodine-131. Pre- and post-treatment imaging with iodine-131 (with consideration of dosimetry for distant metastases) is recommended for suspected or proven metastatic foci

greedy for iodine-131. In patients with known or suspected distant metastatic disease, iodine imaging (iodine-123 or iodine-131) with adequate stimulation of thyroid-stimulating hormone (TSH) (thyroid shrinkage or thyrotropin alfa) should be considered prior to administration of iodine-131 therapy, paying attention to dosage recommendations to avoid the problem of lightheadedness, which may limit the effect of treatment. RAI is not effective in the case of ATC patients [5].

2.6.3 Radiation therapy

Radiation therapy is a treatment modality that uses high doses of radiation to target and destroy cancer cells. In the context of thyroid carcinomas, radiotherapy is not always the first-line treatment, as thyroid cancers tend to be more responsive to surgery and radioiodine therapy. However, there are certain situations in which radiotherapy may be considered for thyroid carcinomas. Among these External Beam Radiation Therapy (EBRT), especially if the cancer has spread to nearby tissues or lymph nodes and cannot be effectively treated with surgery or radioactive iodine, may be considered. Slight improvements in local disease-free survival have been reported after EBRT for selected patients, such as those with extrathyroidal invasion or extensive locoregional node involvement [73]. ERBT is often used as part of the treatment plan for ATC patients to shrink the tumor and relieve symptoms especially if thyroid cancer recurs after initial treatment or if it has metastasized (spread) to other parts of the body. In some cases, when the thyroid cancer is advanced and not responsive to other treatments, radiation therapy may be used to provide palliative care to reduce painful or progressing bone metastases [74]. This aims to relieve symptoms and improve the quality of life for the patient.

2.6.4 Systemic Therapy

Systemic treatment recommendations favor the use of targeted therapeutic options in cases where systemic therapy is indicated. Thyroid cancer guidelines recommend molecular testing

to help make informed decisions about systemic targeted therapy and to determine eligibility for clinical trials. Therapy therefore depends on the result of the molecular test and the mutations involved. The combination of Dabrafenib and Trametinib is an option FDA approved in 2018 for BRAF V600E mutation-positive ATC tumors [75], Larotrectinib or Entrectinib are TRK inhibitors FDA approved for NTRK gene fusion-positive ATC metastatic tumors, based on a pooled analysis of 3 studies (a phase 1 including adults, a phase 1/2 involving children, and a phase 2 involving adolescents and adults) [76,77]. One hundred percent of the thyroid cancers in this study responded to Larotrectinib, with one complete response and 4 partial responses. In 2020, the FDA approved two RET inhibitors, Selpercatinib or Pralsetinib, for RAI refractory RET fusion-positive thyroid cancer requiring systemic therapy [78,79]. That same year, the FDA approved the anti-PD-1 antibody Pembrolizumab for the treatment of solid tumors TMB-H (≥10 mut/Mb), based on the results of a phase II study, which included 2 patients with thyroid cancer [80]. Other recommended regimens include treatment with anthracyclines and taxanes, which are generally not very effective for advanced anaplastic disease, but in some cases may contribute to disease response or maintenance of stable disease [81]. Single-agent Doxorubicin is approved by the FDA for ATC. A randomized trial including 84 patients with advanced thyroid cancer (not limited to ATC) showed an 11.6% complete response rate in patients who received Doxorubicin combined with Cisplatin, compared with a complete response in 0 patients who received single-agent Doxorubicin [82]. Single-agent Paclitaxel may benefit some patients with newly diagnosed ATC; increased survival has been reported in patients with stage IVB disease [83]. Paclitaxel combined with Carboplatin and Docetaxel combined with Doxorubicin are also systemic treatment options for patients with metastatic ATC, but these are category 2B options based on low-quality evidence and lower panel consensus. Given the poor outcomes with current standard therapy, all patients, regardless of surgical resection, should be considered for clinical trials, some of which have tested vascular interference agents such

as Fosbretabulin, Combretastatin A4 phosphate and Crolibulin, or oral PPAR gamma agonists such as Efatutazone or new multitarget therapies including Bevacizumab, Sorafenib, Sunitinib, Imatinib and Pazopanib [84-86] [87] (Figure 2).

SYSTEMIC THERAPY

Systemic Therapy Regimens for Metastatic Disease		
Preferred Regimens		
Dabrafenib/trametinib[2] (*BRAF* V600E mutation positive)	Dabrafenib 150 mg PO and Trametinib 2 mg PO	Twice daily Once daily
Larotrectinib[3] (*NTRK* gene fusion positive)	100 mg PO	Twice daily
Entrectinib[4] (*NTRK* gene fusion positive)	600 mg PO	Once daily
Pralsetinib[5] (*RET* fusion positive)	400 mg PO	Once daily
Selpercatinib[6] (*RET* fusion positive)	120 mg PO (<50 kg) or 160 mg PO (≥50 kg)	Twice daily
Other Recommended Regimens		
Paclitaxel[8]	60–90 mg/m² IV or 135–200 mg/m²	Weekly Every 3–4 weeks
Doxorubicin[8]	20 mg/m² IV or 60–75 mg/m² IV	Weekly Every 3 weeks
Paclitaxel/carboplatin[1] (category 2B)	Paclitaxel 60–100 mg/m², carboplatin AUC 2 IV or Paclitaxel 135–175 mg/m², carboplatin AUC 5–6 IV	Weekly Every 3–4 weeks
Docetaxel/doxorubicin[1] (category 2B)	Docetaxel 60 mg/m² IV, doxorubicin 60 mg/m² IV (with pegfilgrastim) or Docetaxel 20 mg/m² IV, doxorubicin 20 mg/m² IV	Every 3–4 weeks Weekly
Useful in Certain Circumstances		
Doxorubicin/cisplatin[8]	Doxorubicin 60 mg/m² IV, cisplatin 40 mg/m² IV	Every 3 weeks
Pembrolizumab[7] (TMB-H [≥10 mut/Mb])	200 mg IV or 400 mg IV	Every 3 weeks Every 6 weeks

Figure 2. The NCCN Guidelines Version 2.2022 for Systemic Therapy Regimes for Metastatic Thyroid Carcinomas. [Haddad RI, Bischoff L, Ball D, Bernet V, Blomain E, Busaidy NL, Campbell M, Dickson P, Duh QY, Ehya H, Goldner WS, Guo T, Haymart M, Holt S, Hunt JP, Iagaru A, Kandeel F, Lamonica DM, Mandel S, Markovina S, McIver B, Raeburn CD, Rezaee R, Ridge JA, Roth MY, Scheri RP, Shah JP, Sipos JA, Sippel R, Sturgeon C, Wang TN, Wirth LJ, Wong RJ, Yeh M, Cassara CJ, Darlow S. Thyroid Carcinoma, Version 2.2022, NCCN Clinical Practice Guidelines in Oncology. J Natl Compr Canc Netw. 2022 Aug;20(8):925-951. doi: 10.6004/jnccn.2022.0040. PMID: 35948029.].

CHAPTER THREE: BENZYL ISOTHIOCYANATE

3. Benzyl isothiocyanate (BITC)

Isothiocyanates (ITCs) are natural bioactive compounds found as conjugates in cruciferous vegetables of the genus *Brassica* such as broccoli, watercress, brussels sprouts, cabbage, cauliflower, and Japanese radishes [88]. The high content of glucosinolates, which store ITCs in cruciferous vegetables, attributes significant chemo-preventive and anti-carcinogenic effects. In fact, several studies in the literature have found a close correlation between dietary intake of cruciferous vegetables and a reduction in the risk of cancer or cancer complications, mainly attributed to the presence of isothiocyanates (ITCs) in these vegetables [89]. ITCs are the product of the hydrolysis of glucosinolates, which occurs as a defense mechanism against pathogens, by some enzymes of the myrosinasic family [90]. From a chemical point of view, ITCs have a highly electrophilic central carbon atom at the N=C=S structure, therefore, following hydrolysis, they are able to generate reactive oxygen species (ROS) and cause oxidative damage to DNA [91]. The most studied ITCs are allyl isothiocyanate (AITC), benzyl isothiocyanate (BITC), phenethyl isothiocyanate (PEITC) and sulforaphane (SFN) [92]. Of all ITCs, benzyl isothiocyanate (BITC) is the one that has been demonstrated to exert multiple beneficial effects on human health. BITC is one of the main components of alliaria petiolata, pilu oil, watercress, garden cress, and papaya seeds [93]. BITC, with a molecular weight of 149.21 g/mol and the molecular formula C_8H_7NS, is lipophilic and poorly soluble in water, is metabolized through degradation and hepatic conjugation by gluthathione-S-transferase and glutamyl transpeptidase, respectively, and is 62% excreted in the urine as mercapturic acid [94]. The antibacterial, antifungal, and anti-inflammatory properties of BITC are known, but in recent years a growing research interest in the antitumor activity of BITC has been reported. A literature research conducted in 2020 shows a total of 75 publications regarding the anticancer properties of BITC [95], of which 27% investigated the antitumor activity of BITC against breast cancer, followed by pancreatic cancer (19%) and lung cancer (8%),

accumulating *in vitro* and *in vivo* evidence points to the effectiveness of BITC against several types of cancer (Figure 3).

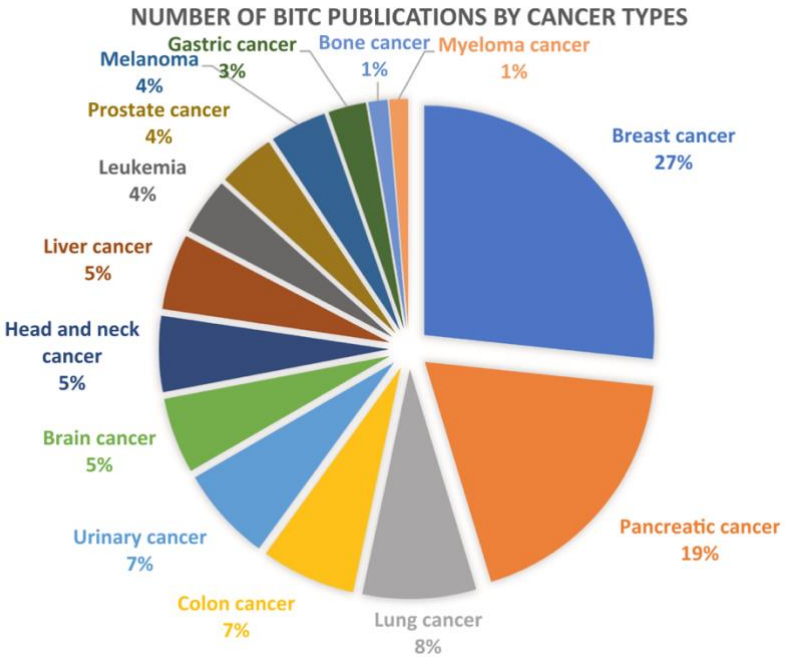

Figure 3. Studies regarding anticancer activity of BITC up to 2020. [Dinh TN, Parat MO, Ong YS, Khaw KY. Anticancer activities of dietary benzyl isothiocyanate: A comprehensive review. Pharmacol Res. 2021 Jul;169:105666. doi: 10.1016/j.phrs.2021.105666. Epub 2021 May 12. PMID: 33989764].

3.1 Signaling pathways involved in BITC mechanism of action

BITC influences several keys signaling pathways which are considered the hallmarks of cancer such as apoptosis, cell proliferation, cell cycle, metastasis, angiogenesis, autophagy.

3.1.1 Cell proliferation and apoptosis

Evidence suggests the ability of BITC to induce cancer cell death through the modulation of several pathways related to apoptosis and cell proliferation. Among the most studied mechanisms, through which BITC can induce programmed cell death, there is certainly the production of reactive oxygen species (ROS), which causes mitochondrial dysfunction through the disruption of membrane integrity and oxidative damage. This mechanism has been observed in a wide variety of tumors: glioblastoma GBM8401 cells [96], cisplatin-resistant oral carcinoma CAR cells [97], gefitinib NCI-H460/G-resistant lung cancer cells [98], estrogen-responsive (MCF-7) and estrogen-independent (MDA-MB-231) human breast cancer cells [99], murine WEHI-3 leukemia cells [100], human melanoma A375. S2 cells [101], pancreatic cancer cells L3.6pL, MiaPaCa2 and Panc-1 [102], human gastric cancer AGS cells [103], human prostate cancer cells DU 145, human prostate cancer cells Rv1 sensitive to hormones and PC3 refractory to hormones [11]. Disruption of mitochondrial integrity caused by BITC is also related to upregulation of the expression of pro-apoptotic proteins Bax, Bad and downregulation of anti-apoptotic proteins Bcl-2 and Bcl-xL. This mechanism of regulation of mitochondrial dynamics through modulation of proteins involved in mitochondrial fusion-fission has been found in estrogen-responsive (MCF-7) and estrogen-independent (MDA-MB-231) human breast cancer cells [104]. Some *in vivo* studies have also demonstrated the tendency to BITC-mediated downregulation of proteins involved in the regulation of mitochondrial dynamics in mammary tumors of MMTV-neu mice and in addition oral administration of BITC increased the expression of the pro-apoptotic proteins caspase-3 and Bax in nude mice affected by GBM 8401 tumor [105]. Furthermore, BITC has been shown to increase the total expression of the tumor suppressor protein p53, its phosphorylation, stabilization and nuclear localization, inducing apoptosis. This mechanism seems to be mediated by the promotion of PUMA (p53 regulated apoptosis modulator)

expression, which facilitates apoptosis through direct interaction with members of the antiapoptotic Bcl-2 family. This mechanism has been studied in MCF7 and MDA-MB-231 breast cancer cells [106], U937 leukemia cells [107] and SCC9 oral squamous cell carcinoma cells. These findings were further validated by *in vivo* studies conducted in SCC9-xenografted SCIID mice, in which intraperitoneal administration of BITC significantly inhibited tumor growth [108]. Another pathway by which BITC regulates apoptotic processes is the suppression of the protein family of inhibitor of apoptosis (IAP) includes scIAP1, XIAP and survivin, key regulators of apoptotic cell death, whose overexpression is associated with the aggressiveness of several types of cancer. BITC has been shown to suppress XIAP and survivin in a lot of *in vitro* studies conducted in glioblastoma GBM8401 cells [109], glioma U87MG cells [110], MCF-7 and MDA-MB-231 breast cancer cells [97], gastric adenocarcinoma AGS cells [103], human liver cancer HLE cells [99], pancreatic cancer cells (L3.6pL, MiaPaCa2 and Panc1) [102], as well as *in vivo* in different mouse tumor models [97]. BITC is capable of triggering the extrinsic apoptotic signaling pathway to induce cancer cell death by inducing activation of TNF-related Aptoptosis-Inducing Ligand (TRAIL) death receptors. These receptors are overexpressed on the surface of cancer cells and represent a potential chemotherapy target, as their activation causes the cleavage of caspase 8 and the activation of caspase 3 initiating cell death [111]. Increases in protein expression levels of death receptors or their ligands (Fas, Fas-L, Fas, FADD and TRAIL) have been observed in glioblastoma GBM8401 cells [109] and in human melanoma cells A375.S2 and AGS cells.

3.1.2 Cell cycle arrest

The eukaryotic cell cycle, intricately regulated by cyclins and cyclin-dependent protein kinases (CDKs) [112], is a meticulously organized series of events comprising the G1, S, G2 and M phases, which are essential for completing the process of cell division and cell replication [113]. Tumor development is often linked to the overexpression of cell cycle

proteins, while the prospect of inhibiting these proteins and enhancing gene expression to control cell proliferation is a promising strategy in preventing cancer progression. Numerous studies have explored the ability of benzyl isothiocyanate (BITC) to hinder cell cycle proteins. BITC has been shown to inhibit the proliferation of mouse TRAMP-C2 prostate cancer cells by inducing G1 cell cycle arrest through downregulation of CDK2, CDK4, cyclin A, and cyclin D1 proteins [17]. BITC also induces substantial G2/M arrest and reduces G1 and S phase populations in human multiple myeloma MM U266 cells [114] and in human pancreatic cancer cells Capan-2 by activating Chk2, Cdc25C, Cdc-2, the cyclin-dependent kinase inhibitor p21Waf1/Cip1, and downregulating cyclin B1 proteins [115]. In addition, BITC regulates the expression levels of G2/M phase proteins, including the downregulation of cyclin A, CDK1, CDC25C and the upregulation of Chk1 and Wee1 proteins in human melanoma cells [101] and enhances the expression of genes associated with inhibition of human glioblastoma cell proliferation, such as CDC-4, CCNG2, CCNA1, CCNYL1, CDK-5, DMTF1 and CCPG1 [96]. In addition, BITC induces cell cycle arrest by modulating cyclin B1 and p21Waf1/Cip1 proteins in glioma cell line U87MG [110].

3.1.3 Angiogenesis

Some preclinical animal studies have demonstrated the ability of BITC to prevent angiogenesis, the process of forming new capillaries from existing blood vessels, which plays a crucial regulatory role in tumor growth by facilitating the transport of nutrients, the removal of waste products, and providing cancer cells with access to the vascular system for diffusion throughout the body. Key proteins in angiogenesis include vascular endothelial growth factor (VEGF) and the VEGF-R2 receptor, the interaction of which promotes endothelial cell proliferation, migration, and differentiation [116]. In an *in vivo* study, BITC significantly inhibited breast cancer angiogenesis by downregulating the expression of the VEGF protein in a xenograft tumor model. This finding is in line with cell-based studies indicating

suppression of VEGF secretion and expression of VEGF receptor protein 2 in MDA-MB-231 cells [117]. Another study showed that oral administration of BITC reduced the volume of GBM 8401 tumors in nude mice [105], while intraperitoneal administration downregulated VE-cadherin, a gene related to angiogenesis, in nude BALB/c mice with U87MG tumor [110].

3.1.4 Metastasis

Among the processes belonging to the metastatic cascade that contribute to the migration of tumor cells from the primary site to other tissues and organs, the epithelial-mesenchymal transition (EMT) emerges as a fundamental mechanism in tumor resistance, invasion and metastasis [118]. During EMT, the epithelial-tumor suppressor cadherin (E-cadherin) is downregulated, while the neural cadherin (N-cadherin) is upregulated, accompanied by overexpression of the intermediate filament protein vimentin, promoting increased cell motility and detachment of tumor cells [119]. Some investigations have revealed the ability of BITC to inhibit EMT by upregulating adherent junction proteins such as E-cadherin and occludin, along with the downregulation of mesenchymal markers such as vimentin and fibronectin in both highly metastatic HN12 head and neck cancer cells [3], both MDA-MB-231 and MCF-7 breast cancer cells [12,120]. Treatment with BITC has also been shown to inhibit leptin-induced clonogenicity, an adiponectin responsible for three-dimensional colony formation, invasion and migration of MDA-MB-231 and MCF-7 breast cancer cells [121]. Further studies have underlined the effect of BITC in reducing the formation of skeletal metastases in breast cancer patients through modulation of the RANK receptor and its ligand RANKL. BITC, in fact, reduced the number of breast cancer-induced osteoclasts and attenuated osteoclast differentiation by suppressing sRANKL expression in MCF-7, MDA-MB-231 and RAW264 breast cancer cells [122]. Tumor cell metastases often result from impaired cell-extracellular matrix (ECM) adhesion, disrupted intercellular interactions, and ECM degradation. This can be attributed to the overexpression of matrix metalloproteinases

(MMPs) such as MMP-2 and MMP-9 and urokinase plasminogen activator (u-PA), which leads to degradation of ECM components and release of metastasis-inducing growth factors from the ECM [123]. The activity of MMP-9 is regulated by transcription factors such as activator protein AP-1, nuclear factor NF-κB, and focal adhesion kinase (FAK) [124]. BITC inhibited tumor growth and metastasis in U2OS osteosarcoma cells by suppressing MMP-9 protein expression through inhibition of TPA-induced FAK phosphorylation [125].

3.1.5 MAPK signaling

Mitogen-activated protein kinases (MAPKs) are serine/threonine-specific protein kinases, present in the cell cytoplasm, which by activating and phosphorylating downstream cytosolic proteins, govern essential intracellular pathways and play crucial roles in cellular processes such as cell growth, differentiation, and apoptosis [126]. Dysregulation of MAPK signaling pathways is implicated in proliferation, angiogenesis, and metastasis of cancer cells, with hyperactivation of proteins such as extracellular signal-regulated kinase (ERK), N-terminal kinase c-Jun (JNK), p38 [127]. Several papers have reported that BITC modulates apoptosis, cell cycle arrest and angiogenesis through interaction with MAPK signaling pathways. For example, BITC induced apoptotic cell death through p38 MAPK activation in head and neck squamous cell carcinoma (1483 and UM-22B), while a structurally similar compound, phenyl isothiocyanate (PITC), showed no comparable effects, suggesting the importance of BITC's unique chemical structure in its antitumor ability [128]. Activation of this signaling pathway has been reported in Capan-2 pancreatic ductal adenocarcinoma cells [129], MDA-MB-231 and MCF-7 breast cancer cells [130], where exposure to BITC significantly increased phosphorylation of ERK, JNK and p38 inducing apoptosis and in some cases cell cycle arrest. Beyond apoptosis, the ERK/MAPK pathway can modulate transcription factors involved in matrix metalloproteinase (MMP) synthesis and activation, which degrade the extracellular matrix and increase tumor cell metastasis. BITC inhibited ERK1/2 in AGS human gastric

cancer cells [131], leading to a reduction in MMP-2 and MMP-9 protein and mRNA expression, with a consequent decrease in migration and invasion. Similar inhibitory effects on tumor metastases were observed in U2OS osteosarcoma cells [125], SK-Hep1 liver cancer cells [132], and human colon cancer HT29 cells [18], where BITC suppressed MMP-2 and MMP-9 protein expression by downregulating MAPK signaling pathways.

3.1.6 Autophagy

Autophagy is an intracellular process that contributes to the maintenance of cellular and energy homeostasis by protecting the cell through the degradation and elimination of damaged organelles and obsolete proteins, playing an adaptive role in development and cell death under stressful conditions, such as nutrient deprivation. Of all signaling pathways, the one with which BITC has been shown to interact the most is autophagy**.** BITC reduced viability and have shown to induce autophagy-related LC3-II protein processing in Rv1 and PC3 prostate carcinoma cells through inhibition of the mammalian target of rapamycin (mTOR) kinase signaling pathway [19]. Similar effects were reported in human colorectal carcinoma HCT-116 cells, where BITC upregulated autophagy symbol proteins, including LC3BII and LAMP1 [15]. Likewise, induction of autophagy by BITC was demonstrated in A549, H661 and SK-MES-1 lung cancer cell lines, through accumulation of LC3-II protein and increased expression of Atg5, which led to inhibition of tumor growth [14]. Also in breast cancer cells MDA-MB-231, MCF-7, MDA-MB-468, BT-474 and BRI-JM04, BITC was able to activate autophagy both in *vitro* and *in vivo* by LC3 cleavage and suppression of p62 and mTOR expression [133]. This effect appears to be mediated by increased expression and acetylation of forkhead box protein O1 (FOXO1), a key regulator of autophagy in breast cancer cells. Similarly, in a xenograft model of mouse BxPC-3 pancreatic adenocarcinoma, oral administration of BITC was shown to suppress tumor growth through inhibition of the PI3K/AKT pathway and phosphorylation of downstream transcription factor FOXO [134]. In

addition, BITC inhibited the phosphorylation of AKT through upregulation of the tumor suppressor phosphatase and tensin homologue deleted on chromosome 10 (PTEN) to suppress the growth of human glioma U87MG cells [110]. PTEN, through the dephosphorylation of the lipid phosphatidylinositol-3,4,5-triphospate (PIP3), plays a role in the negative regulation and homeostatic maintenance of phosphatidylinositol-3 kinase (PI3K) cascades at the time of activation [135]. PIP3 modulates cellular physiological processes through its downstream signaling proteins: protein kinase B (AKT), and mammalian target rapamycin (mTOR). The mutation or loss of PTEN causes the alteration of AKT/mTOR functions, which is consequently responsible for the induction of tumorigenesis in most human tumors [136]. The ability of BITC to exert antitumor properties by regulation of PTEN and interaction with the PI3K/AKT pathway has been demonstrated in conducted work of highly metastatic L9981 lung cancer cells and HCT-116 colon cancer cells [137,138].

3.1.7 Cellular Senescence

Cellular senescence is a condition of quiescence between life and death that cells can be subjected in response to different internal and external stimuli, such as dysfunctional telomeres, mitogenic signals, oxidative stress, oncogene activation, ionizing radiation, treatment with anti-cancer therapies, which can cause persistent DNA damage and trigger permanent cell cycle arrest [139,140]. This stable cell cycle arrest is correlated with an augmented level of cell cycle inhibitors, including p16INK4a, p21CIP1, and p27 and it limits cell proliferative lifespan [141]. Thus, thanks to its antiproliferative effects, senescence is considered a potent antitumor mechanism. This tumor-suppressive function of senescence has paved the way for new senescence-enhancing treatments for cancer therapy, a process called prosescence therapy [142]. Senescent cells undergo characteristic changes, including chromatin rearrangement, cell cycle arrest and increased senescence-associated β-galactosidase (SA-β-gal) activity. Features of senescence is also the secretion of pro-

inflammatory cytokines, growth factors and matrix metalloproteinases (MMPs), collectively termed the senescence-associated secretory phenotype (SASP), which is primarily responsible for the paracrine effects of senescent cells, by reinforcing the senescent growth arrest and/or by promoting immune surveillance [143,144]. Some isothiocyanates including Phenethyl isothiocyanate (PEITC) have been shown to have a prosenescence action mediated by modulation of senescence effectors p16, p53 and p21 and increased staining for SA-β-Gal in tumor cells at concentrations ranging from 4 μM to 20 μM [145-149]. PEITC also downregulated telomerase in cervical cancer cells (HeLa) [148]. Among the isothiocyanates, sulforaphane (SFN) is also capable of inducing senescence through the production of ROS. SFN appears to interfere with GSH/GSSG equilibrium to promote ROS generation, which in turn could trigger ROS-mTOR-dependent autophagy inhibition and induction of paracrine senescence in ESCC cells [150]. Although the SASP of senescent cancer cells is initially tumor suppressive in the long term it creates a chronic inflammatory microenvironment that supports cancer growth. To achieve better antitumor responses by SASP factors, senescence-inducing therapies can be combined with senolytic treatments [151]. This so-called, "one-two punch" approach may be most effective in eliminating cancer cells, in which the first drug induces cancer cells to be senescent cells that are killed by the second senolytic agents. To date, there is little evidence on the effect of BITC in modulating the senescent response, therefore this thesis work aims to also investigate this aspect.

3.1.8 MicroRNAs

MicroRNAs constitute a class of small non-coding RNAs (ncRNAs), whose fundamental role is to negatively regulate gene expression at the post-transcriptional level. They act as control elements of complex regulatory pathways underlying many fundamental cellular processes, such as the cell cycle, proliferation, differentiation and apoptosis [152]. Therefore, changes in the activity of miRNAs caused by chromosomal aberrations, genetic mutations, and epigenetic

changes, have been found in several diseases, including cancer [153]. In this respect, there is now a large body of evidence demonstrating that more than 60% of the human genes are regulated by miRNAs, which, in dependence on the tissue or organ and target genes, might act as tumor suppressors or oncogenes [154]. The importance of miRNAs in tumors is highlighted in the literature by numerous studies showing their expression profiles in a wide variety of human carcinomas. The involvement of miRNAs in TC carcinogenesis has recently changed the paradigm for the discovery of biomarkers of clinical interest in these neoplasms, suggesting how these small ncRNAs can be used to develop, refine, or strengthen diagnostic strategies and therapeutic choice in TC [155]. MiR-99a has recently been reported as a tumor suppressor gene in various human cancers, including anaplastic (ATC) and papillary (PTC) thyroid carcinomas. Indeed, some studies suggest the involvement of miR-99a in the inhibition of cell proliferation and reduction of thyroid cancer progression through targeting of the mTOR/p-4E-BP1/p-S6K1 pathway [156,157]. BITC has shown anticancer effects through upregulation of miR-99a mediated by modulation of the ERK signaling pathway and mitogen-activated protein kinase (MAPK) in bladder cancer (BC) [158]. Another study demonstrated that BITC treatment inhibited expression of prosurvival proteins IGF1R, mTOR and FGFR3 by upregulation of miR-99a in human BC cells [13].

CHAPTER FOUR: AIM OF THE THESIS

4. Aim of the thesis

Based on the promising antitumor capabilities that BITC has demonstrated over the years in several preclinical studies conducted on different types of tumors, the present PhD project aims to apply this knowledge also to anaplastic thyroid carcinoma (ATC) in order to shed light on the mechanisms of action underlying the effects responsible for tumor suppression. This evaluation was conducted through both *in vitro* and *in vivo* models of ATC, emphasizing the modulation by BITC of signaling pathways defined as hallmarks of cancer, including. apoptosis, inflammation, autophagy, mesenchymal epithelial transition, and cellular senescence. Given the limited treatment options and challenges of drug resistance in ATC, there is a growing interest in finding effective strategies for cancer prevention or treatments, including the use of natural, synthetic, or biologic agents to prevent, suppress, or reverse the early stages of carcinogenesis. Knowledge at the molecular level of the multiple pathways that play a critical role in the fundamental stages of carcinogenesis initiation, promotion and progression have significantly improved the prevention of certain types of cancer including breast, colon and prostate cancer, so this research could represent a significant effort to address a challenging and deadly type of cancer such as ATC. Understanding how BITC influences ATC tumor progression and identifying relevant signaling pathways can potentially lead to the development of innovative and more effective therapeutic approaches for ATC patients, offering hope for improved outcomes and quality of life.

CHAPTER FIVE: MATERIALS AND METHODS

5. Materials and Methods

5.1In vitro studies

5.1.1 Materials

BITC was obtained from Sigma–Aldrich Company (Milan, Italy, cat. 252492). All the chemical reagents used for the studies were of the highest commercial grade available. All stock solutions were made in nonpyrogenic saline (0.9% NaCl; Baxter Healthcare Ltd., Thetford, Norfolk, UK).

5.1.2 Cell cultures

Human TC cell lines FTC-133, K1 and 8305C were obtained from ATCC American Type Culture Collection, Rockville, MD, USA). Cells were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA cat. R8758) supplemented with 10% fetal bovine serum (FBS, Life Technologies, Gibco®; Carlsbad, CA, USA), 100 U/ml of penicillin and 100 μg/ml of streptomycin. All cell lines were maintained in incubators at 37 ° C with 5% CO_2.

5.1.3 Cell viability (MTT Assay)

Cell viability of FTC-133, K1 and 8305C cells was evaluated using a mitochondria-dependent dye for live cells (tetrazolium dye; MTT) (M5655; Sigma-Aldrich). Cells were plated on 96-well plates at a density of 4×10^4 cells/well to a final volume of 150 μl. After 24 hrs, FTC-133, K1 and 8305C cells were treated with BITC (Sigma-Aldrich®) for 24 hrs at increasing concentrations 1 μM, 10 μM, 30 μM, 50 μM, 100 μM, 200 μM and 300 μM dissolved in basal medium. After 24 hrs cell were incubated at 37°C with MTT (0.2 mg/mL) for 1 h, the medium was removed by aspiration and then cells were lysed with DMSO (100 μl). The extent of

reduction of MTT to formazan was quantified by measurement of optical density at 540 nm (OD540) with a microplate reader as previously described [159].

5.1.4 Experimental groups

1) Control group: TC cell lines: FTC-133, K1 and 8305C;
2) BITC 30 μM group: FTC-133, K1 and 8305C cells were treated with BITC 30 μM for 24 hrs;
3) BITC 100 μM group: FTC-133, K1 and 8305C cells were treated with BITC 100 μM for 24 hrs;
4) BITC 200 μM group: FTC-133, K1 and 8305C cells were treated with BITC 200 μM for 24 hrs;

For other analysis we continued to analyze only BITC 30 μM, 100 μM and 200 μM because represented the most cytotoxic concentrations revealed by MTT assay. Moreover, since BITC showed similar effects on cell viability in all three cell lines, we decided to continue to analyze the effect of BITC only on 8305C cell line because it represents one of the most frequently used cell line in the field of ATC.

5.1.5 Western Blot Analysis

Western blot analysis was performed as previously described [160]. For cell lysates, 8305C cells were washed twice with ice-cold phosphate buffered saline (PBS), collected and resuspended in lysis buffer containing 20 mM Tris-HCl pH 7.5, 10 mM NaF, 150 μl of NaCl, 1% Nonidet P-40 and protease cocktail of inhibitors (Catalog No. 11836153001; Roche, Switzerland). After 40 minutes, cell lysates were centrifuged at 12,000 rpm for 15 minutes at 4 °C. Protein concentration was estimated using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as a standard. The samples were then heated to 95 ° C for 5 minutes and equal amounts of proteins were separated by 10% -15% sodium dodecyl

sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a membrane of polyvinylidene difluoride (PVDF) (Immobilon-P, catalog # 88018; ThermoFisher Scientific). The following primary antibodies were used: anti-BCL-2 (1: 500; sc-7382; Santa Cruz Biotechnology), anti-BID (1:500; sc-11423; Santa Cruz Biotechnology), anti-BAX (1:500; sc-20067; Santa Cruz Biotechnology), anti-pmTOR (1:500; sc-517464; Santa Cruz Biotechnology), anti-MAP LC3 (1:500; sc-398822; Santa Cruz Biotechnology), anti-p62 (1:500; sc-48402; Santa Cruz Biotechnology), anti-LAMP2 (1:500; MA1-205 Invitrogen), anti-pP38 (1:500; sc-7973; Santa Cruz Biotechnology), anti-TRAF6 (1:500; sc-8409; Santa Cruz Biotechnology). The antibody dilutions were made in PBS / 5% w / v skimmed milk powder / 0.1% Tween-20 (PMT) and the membranes were incubated overnight at 4° C. The membranes were then incubated with a secondary antibody (1: 2000; Jackson ImmunoResearch, West Grove, PA, USA) for 1 hour at room temperature. To ensure that the stains were loaded with equal amounts of protein lysate, they were also incubated with β-actin antibody (cytosolic fraction 1: 500; sc-47778; Santa Cruz Biotechnology) or laminin A/C (nuclear fraction 1: 500; sc-376248; Santa Cruz Biotechnology). The signals were detected with enhanced chemiluminescence (ECL) detection system mixture (Thermo Fisher, Waltham, MA, USA).

5.1.6 Wound healing assay (Scratch test)

The effects of BITC on 8305C cell migration was performed by the Wound healing assay (Scratch test) as previously described [161]. Briefly, 2×10^6 8305C cells were plated on 60 mm plates (Corning Cell Culture, Tewksuby, MA, USA) in a final volume of 2 ml to obtain a confluent monolayer. 24 h later, cell monolayer was scratched creating a straight line using a p200 pipette tip. After removing debris from each plate, cells were treated with increasing concentrations of BITC (30 μM, 100 μM and 200 μM) for 48 hours. For the control group, cells were treated with normal culture medium. Finally, to record the wound width and therefore the migratory ability of the cells, photos of each plate were acquired through a phase contrast

microscope at 0, 24 and 48 hours. At the end of the experiment the cell migration rate was analyzed using Image J software.

5.1.7 Colony formation assay

For the colony formation assay 8305C cells were grown in six-well plates at 1,000 cells per well, and then treated with different concentrations of BITC (30 μM, 100 μM and 200 μM) or with solvent alone as a control. After 24 h of treatment, the wells were washed with PBS and incubated with RPMI-1640 medium supplemented with 10% FBS. After incubation for 10 days, the cells were washed twice with PBS and stained with 0.1% (w/v) crystal violet. The stained cells were imaged using a bright-field microscope (Zeiss) [162].

5.1.8 Enzyme-Linked Immunosorbent Assay (ELISA) for IL-17 and IL-12p70

To evaluate the inflammatory response, the levels of IL-17 (ELISA kit for IL-17, SEA063Hu Cloud-Clone Corp.) and IL-12p70 (Human IL-12p70 ELISA kit, KE00019, Proteintech) were measured in cell lysates collected by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions. Briefly, 100 μl of standards and cell lysates were added to the appropriate wells and incubated for 1 hour at 37 °C. Then, 100 μl of 1x Detection Reagent A was added to each well and the plate was incubated for 1 hour at room 37 °C. The solution was then discarded, and 4 washes were performed with 1X Wash Solution. Later 100 μl of streptavidin solution was added to each well and the plate was incubated for other 30 minutes. After repeated washes, 90 μL of Substrate Solution was added to each well and the plate was incubated for 10-20 min at 37 °C protecting it from light. After that, 50 μL of Stop Solution was added to each well and the absorbance was read immediately using microplate reader at 450 nm.

5.1.9 Proliferation assays

Proliferation assay in 8305C cell line was performed by plating 1×10^4 cells per well of a 96-well plate in at least triplicate. Proliferation was monitored and analyzed by using Incucyte S3 in vitro system (Essenbioscience)[163], but also using Crystal Violet assay. For each time point, cells were washed twice with 1x PBS followed by 10 mins fixation using 10% Formalin (Thermo Fisher Scientific, 5701). After fixation, formalin was aspired followed by 2 washes with 1x PBS and thereafter stained with crystal violet (Sigma, V5265) for 15 mins. Afterwards, the crystal violet staining solution was washed with distilled water and allowed to dry overnight. Representative images of the plates were obtained using an image scanner and the cells were destained using 10% acetic acid solution on the shaker at room temperature for 30-40 mins. The crystal violet dye extracted from the cells was measured using spectrophotometer at 590 nm [164].

5.1.10 Condition media assay

Cell supernatants were harvested and spun down at 453 g for 10 min and the supernatant was filtered using 0.22 mm filters. Conditioned medium was administered to parental cells at time zero or 48 h prior the assay for proliferation and β-galactosidase (SA-β-gal) assays, respectively. The conditioned medium in all the experiments was normalized based on the number of cells present in the well at the moment of the harvesting [163].

5.1.11 Senescence associated β-galactosidase (SA-β-gal) assay

For the *in vitro* evaluation of senescent cells, SA- β -gal staining was performed using Senescence β-Galactosidase Staining Kit (Cell Signaling Technology, Cat. No 9860) according to the manufacturer's instructions [163].

5.1.12 Quantitative real-time PCR (RT-qPCR)

RNA extraction from 8305C cells was performed using Trizol (Ambion, life technologies, 15596026), and the cDNA was obtained using ImPROM II kit (Promega, A3800) following the manufacturer's instructions. For RT-qPCR Gotaq® qPCR Master Mix, Promega® (A6002) on Step One Real-Time PCR systems (Applied Biosystems) was used. Expression levels were calculated using the ΔΔCT method [163].

5.2 *In vivo* studies

5.2.1 Animals

For *in vivo* studies BALB/c nude male mice (25-30 g; 6-8 weeks of age) were used and purchased from Envigo (Milan, Italy). Mice were safely placed in a controlled environment and were fed with a standard diet and water ad libitum under pathogen-free conditions with a 12 h light/12 h dark. Animal study was approved by the University of Messina in accordance with Italian regulations on the use of animals (D.M.116192) and Council Regulation regulations (EEC) (O.J. of E.C. L 358/1 12/18/1986).

5.2.2 Orthotopic model of ATC

For the orthotopic model of ATC, BALB/c-nu/nu mice will receive an injection of 5 $x10^5$ 8305C cells, resuspended in 50 μl of saline, into the right lobe of thyroid using an insulin syringe with a 28G 1/2 needle, after anesthetizing the animals with 3% isoflurane. At the end of the procedure the animals were monitored daily and weighed periodically to assess overall health. 13 days later the injection, animals were treated with oral administration of BITC at different doses (5, 10 and 30 mg/kg) for 2 weeks. At the end of the experiments the animals were euthanized and thyroids harvested, weighed and analyzed [165].

The mice were randomly divided into five experimental groups, as described below:

1) SHAM group (8): oral administration of saline;
2) ATC group (8): mice that received tumor cell inoculation, orally administrated with saline;
3) ATC + BITC 5 mg/kg group (8): mice that received tumor cells inoculation, orally administrated with BITC at the dose of 5 mg/kg;
4) ATC + BITC 10 mg/kg group (8): mice that received tumor cells inoculation, orally administrated with BITC at the dose of 10 mg/kg.
5) ATC + BITC 30 mg/kg group (8): mice that received tumor cells inoculation, orally administrated with BITC at the dose of 30 mg/kg.

5.2.3 Histological evaluation

Histological evaluation was performed as previously described [160]. Thyroid samples were quickly removed and fixed with 10% buffered formalin for at least 24 h at room temperature. After dehydration in graded ethanol and xylene, samples were embedded in paraffin and sectioned at 7 μm thickness. After staining with hematoxylin and eosin, sections were observed by an optical microscope (Nikon Eclipse Ci-L microscope). The histological results are shown at 20× magnification (bar scale at 50 μm). Histological analyses were conducted in a blinded manner.

5.2.4 Masson's Trichrome staining

Morphological evaluation of tumors was performed on 5 μm sections stained using the Masson's trichrome kit (Bio-Optica cat: 04-010802), according to manufacturer instructions. The images were acquired using a Nikon Eclipse Ci-L microscope at the magnification of 20x (50 μm of the bar scale)[166].

5.2.5 Immunohistochemistry assay

Immunohistochemical localization for E-Cadherin (1:100; sc-8426 Santa Cruz Biotechnology, Dallas, TX), N-Cadherin (1:100; sc-59987 Santa Cruz Biotechnology, Dallas, TX), and S100 antibodies (1:100; sc-53438 Santa Cruz Biotechnology, Dallas, TX), was made as previously described [167]. The images were shown at a magnification of 20x (50 μm of the bar scale) using a Nikon Eclipse Ci-L.

5.2.6 Western Blot Analysis

Protein levels in thyroid samples were quantified as previously described [168]. Cytosolic proteins were prepared and separated electrophoretically to be transferred to nitrocellulose membranes. Membranes were blocked with 5% (w/v) dried nonfat milk in buffered saline (PM) for 45 min at room temperature and subsequently probed with specific antibodies: anti-BID (1: 500; sc-11423; Santa Cruz Biotechnology), anti-BCL-2 (1: 500; sc-7382; Santa Cruz Biotechnology), anti-BAD (1:500; sc-8044; Santa Cruz Biotechnology), anti-pmTOR (1:500; sc-517464; Santa Cruz Biotechnology), anti-p62 (1:500; sc-48402; Santa Cruz Biotechnology), anti-PTEN (1:500; sc-7974; Santa Cruz Biotechnology) and anti-SOS1 (1:500; sc-17793; Santa Cruz Biotechnology) in 1× PBS, 5% w/v dried nonfat milk and 0.1% Tween-20 (PMT) at 4 °C overnight. Membranes were incubated with peroxidase-conjugated goat anti-mouse IgG secondary antibody (1:2000, Jackson ImmunoResearch, West Grove, PA, USA) or peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:5000, Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature. To establish that blots were loaded with equal amounts of proteins, they were also incubated in the presence of the antibody against β-actin protein (sc-8432, 1:500; Santa Cruz Biotechnology, Dallas, TX, USA). An enhanced chemiluminescence (ECL) reagent was used for signal detection according to the

manufacturer's instructions (Thermo, Waltham, MO, USA, cat# 457). The relative expression of protein bands was quantified by densitometry with Bio-Rad ChemiDoc XRS+ software and standardized to β-actin levels as an internal control.

5.2.7 Statistical Analysis

All values are expressed as mean ± standard deviation (SD) of N observations. Each analysis was performed three times with three samples replicates for each one. Data obtained were analyzed by one-way analysis of variance (ANOVA) followed by a Bonferroni post hoc test for multiple comparisons. A value of $p < 0.05$ was considered significant.

CHAPTER SIX: RESULTS

6. Results

6.1 In vitro results

6.1.1 BITC reduces TC Cell Viability

MTT assay was used to assess FTC-133, 8305C, and K1 cell viability following 24 h of treatment with BITC at different concentrations (1 μM, 10 μM, 30 μM, 50 μM, 100 μM, 200 μM and 300 μM). Our results show that BITC treatments were able to decrease cells viability in a concentration dependent manner as showed in Figure 1. (A, B, C). Based on the MTT results, we decided to investigate in further analysis only BITC at concentrations of 30 μM, 100 μM and 200 μM because these represented the most effective concentrations in the reduction of cell proliferation as demonstrated by the colony formation assay (Figure 1E). Moreover, since BITC showed better effects on cell viability in 8305C cell line, we decided to continue to investigate its effect on only the anaplastic carcinoma cell line, because it represents the most aggressive type of thyroid carcinoma with poor prognosis and with limited therapeutic possibilities.

6.1.2 BITC reduces cell migration and proliferation

The effect of BITC on 8305C cell migration was evaluated using an *in vitro* wound healing test. Confluent cells were scratched and then subjected to BITC treatment for 48 hours. The images were acquired, and the percentage of cells migrated to the scratched area was calculated. Our results showed that BITC led to a marked reduction in the number of cells migrating to the scratched area, particularly at the concentration of 100 μM and 200 μM after 48 hours of treatment (Figure 1.D). The ability of 8305C cells to form colonies was evaluated

following treatment with 30 µM, 200 µM and 300 µM of BITC. The results of 0.1% (w/v) crystal violet staining suggested that BITC significantly inhibited colony formation (Figure 1.E).

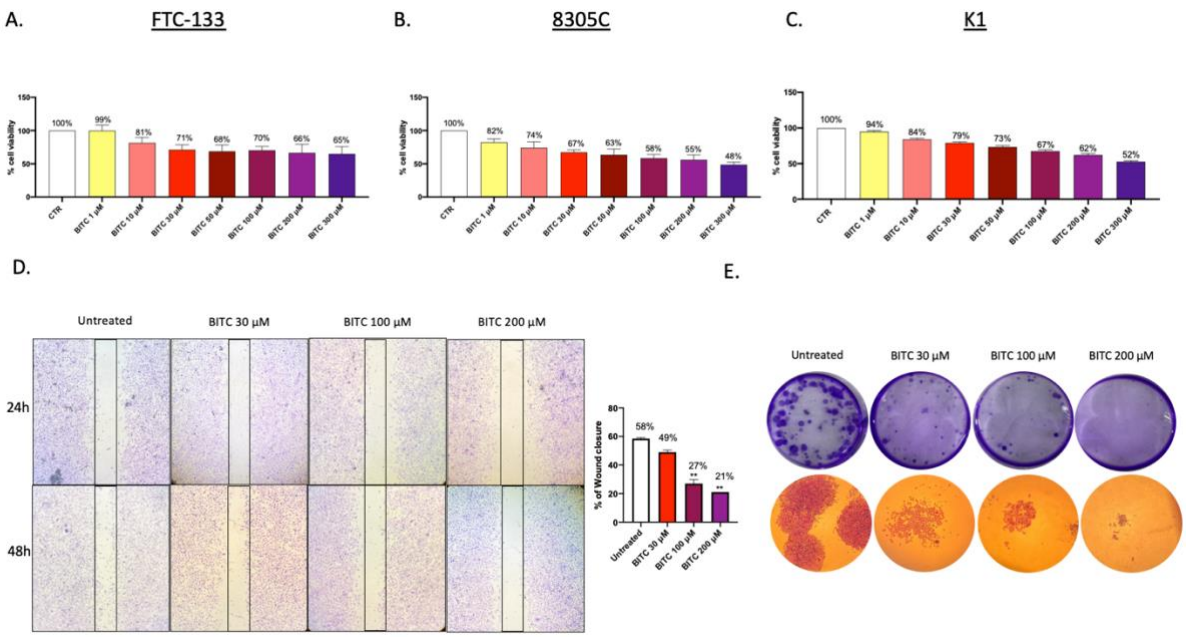

Figure 1. Effect of BITC on cell viability, migration and colony formation. BITC treatment was able to significantly reduce cell viability in a concentration dependent manner on FTC-133 cells **(A)**, 8305C cells **(B)** and K1 cells **(C)**. The Wound healing assay (Scratch test) revealed a significant reduction in the number of cells migrating to the scratched area, following 48 hours of BITC treatment at the concentrations of 100 µM and 200 µM **(D)**. ** $p < 0.01$ vs. Untreated 8305C cells. The colony formation assay of 8305C cells treated with 30 µM, 100 µM, 200 µM BITC for 24h, followed with 0.1% (w/v) crystal violet staining of attached cells after 10 days, showed a significant reduction of colonies formation compared to untreated 8305C cells **(E)**. ***$p < 0.001$.

6.1.3 BITC modulates autophagy markers

To examine the effect of BITC on the autophagy of 8305C cells, we measured the expression of autophagy-related proteins (p-mTOR, p62, MAP LC3 and LAMP2) by Western blot analysis. Our data showed that the expression of p-mTOR and p62 proteins were significantly reduced after the treatment with 30 μM, 100 μM and 200 μM BITC (Figure 2.B-C). At the same time, we demonstrate an upregulation of MAP LC3 and LAMP2 in 8305C cells following the treatment with 200 μM BITC (Figure 2.D-E).

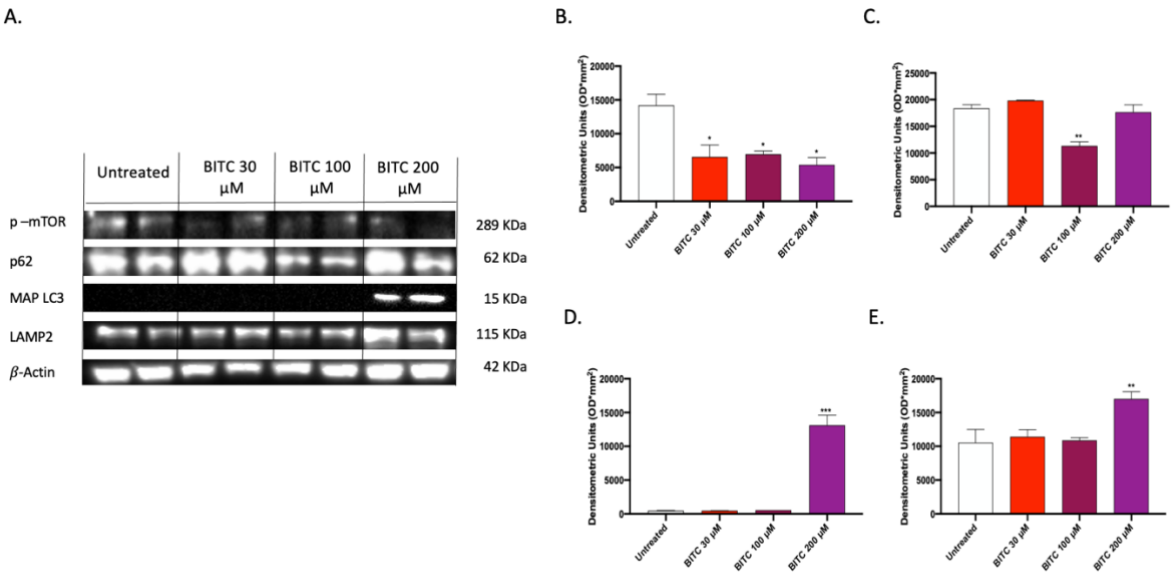

Figure 2. Effect of BITC on autophagy markers expression in 8305C cells. The blots revealed a significant modulation of autophagy markers expression following BITC treatment **(A)**. BITC at the concentrations of 30 μM, 100 μM, and 200 μM was able to significantly reduce the expression of p-mTOR **(B);** * $p < 0,05$ vs untreated 8305C cells. BITC at the concentration of 100 μM causes a significant reduction of p62 expression **(C). ;** ** $p < 0.01$

vs untreated 8305C cells. Treatment with 200 μM BITC was also able to increase the expression of MAPLC3 **(D)** and LAMP2 **(E)** markers compared to untreated 8305C cells; *** $p < 0.001$ vs untreated; ** $p < 0.01$ vs untreated. Our data are the result of three experimental replicates.

6.1.4 BITC modulates apoptosis pathway

Western blot analysis was performed in order to evaluate the effect of BITC on the expression of apoptotic markers such as BAX, BID and BCL-2. Our results showed that BITC, at higher concentrations of 200 μM, significantly improved the levels of pro-apoptotic proteins BAX and BID (Figure 3.C-D), and it was also able to reduce the expression of the anti-apoptotic BCL-2 protein (Figure 3.B). In addition, BITC at the concentration of 200 μM was capable to induce the activation of p38 promoting 8305C cell death (Figure 3.E).

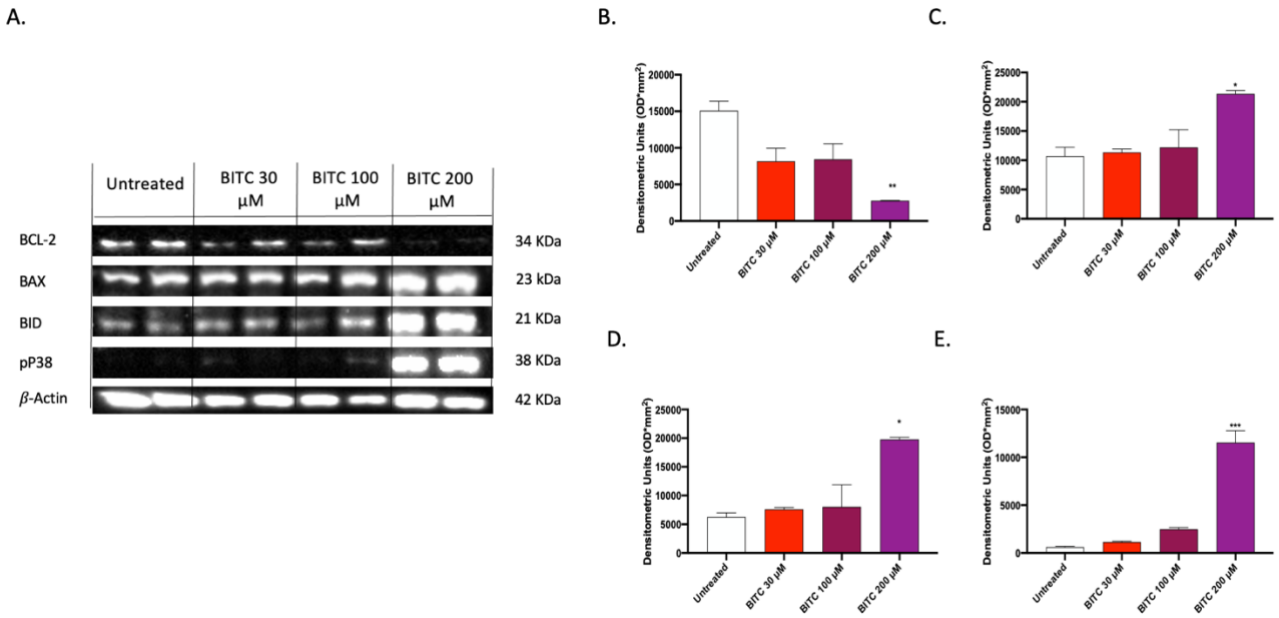

Figure 3. Effect of BITC on apoptosis markers expression in 8305C cells. The blots revealed a significant modulation of apoptosis markers expression following BITC treatment **(A)**. BITC at the concentration of 200 μM was able to significantly reduce the expression of BCL-2 **(B)** and to increase the expression of BAX **(C)** BID **(D)** and pP38 **(E)** markers compared to untreated 8305C cells; *** $p < 0.001$ vs untreated; ** $p < 0.01$ vs untreated; * $p < 0.05$ vs untreated.

6.1.5 BITC modulates inflammation – associated cancer

Previous studies have indicated that cancer may be promoted and/or exacerbated by inflammation and infection. The cytokines produced by activated innate immune cells that stimulate tumor growth and progression are considered as important components in this process [169]. In this contest our data showed an important down-regulation of TRAF6, IL-17 and IL-12p70 in 8305C cells after the treatment with BITC compared to the control (Figure 4.), suggesting its role in maintaining the tissue barrier functions necessary for host defense.

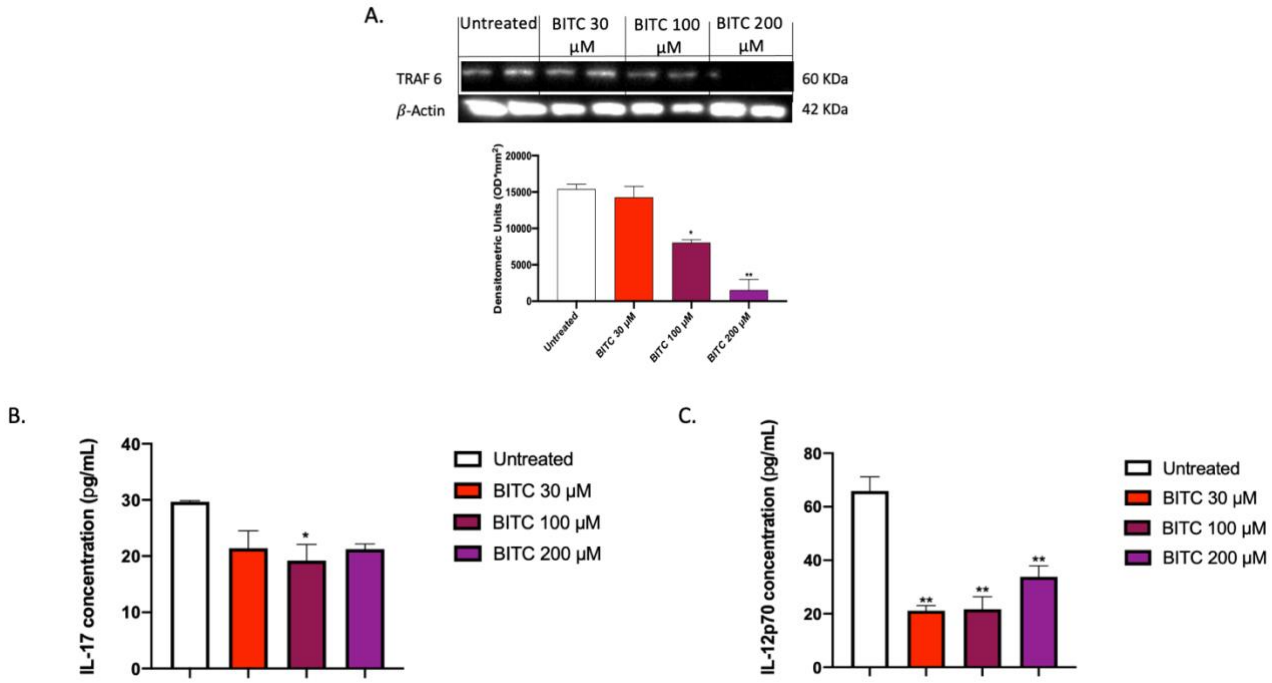

Figure 4. Effect of BITC on inflammation – associated cancer in 8305C cells. BITC treatments at the concentrations of 100 µM and 200 µM was able to significantly reduce the expression of TRAF6, compared to untreated 8305C cells **(A)**; ** $p < 0.01$ vs untreated; * $p < 0.05$ vs untreated. The enzyme-linked immunosorbent assay (ELISA) revealed a significant decrease of IL-12p70, following BITC treatments at all the concentrations **(D)** and also of IL-17 levels, especially at the concentration of 100 µM **(B)** * $p < 0.05$ vs. untreated ; ** $p < 0.01$ vs untreated .

6.2 *In vivo* results

6.2.1 BITC reduces tumor growth on ATC orthotopic model

To evaluate the effect of BITC on the growth of ATC cells *in vivo,* the 8305C orthotopic model was established in nude mice. The histological analysis of the ATC group showed

features of a high-grade malignant neoplasm characterized by high-grade nuclear atypia, marked cellular pleomorphism, necrosis, and significant neutrophil infiltration compared to the sham group [170]. Our results demonstrate that the treatment with BITC at doses of 10 and 30 mg/kg was able to significantly reduce these pathological features as showed in Figure 5. No important changes in the animals' body weight was showed during the experiments (Figure 5 F.).

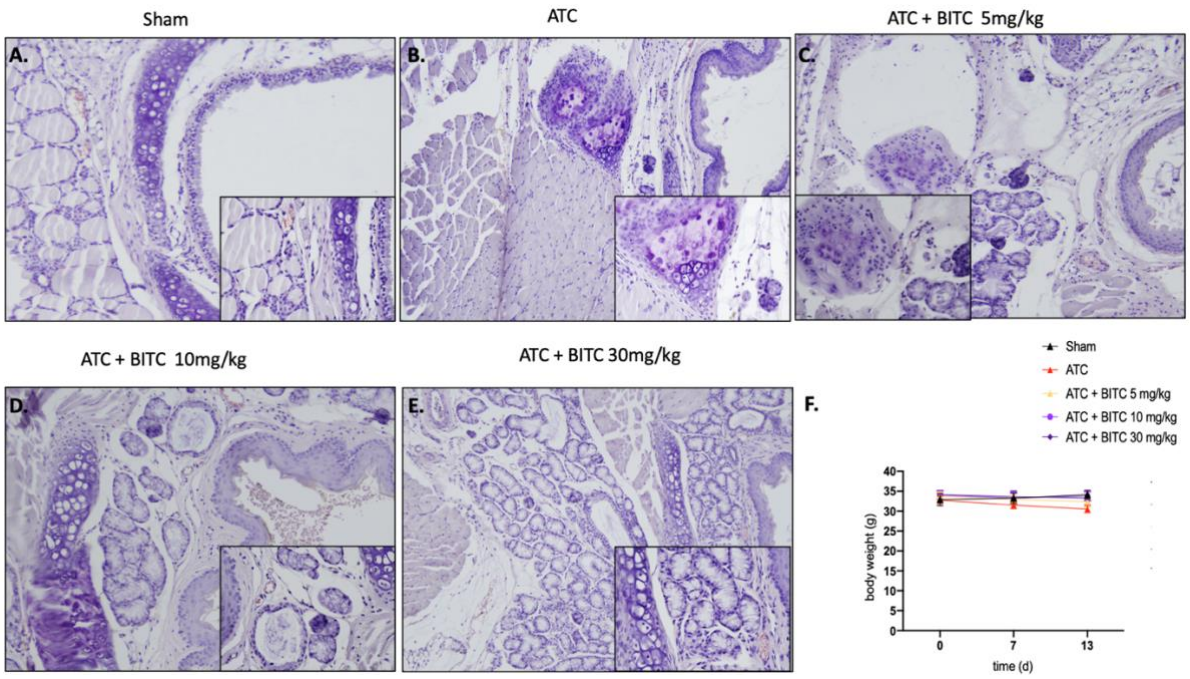

Figure 5. Effect of BITC on tumor growth in an orthotopic model of ATC. BITC treatments at the concentrations of 10 mg/kg **(D)** and 30 mg/kg **(E)** were able to reduce features of a high-grade malignant neoplasm such as high-grade nuclear atypia, marked cellular pleomorphism, necrosis, and neutrophil infiltration compared to the sham group **(A)**. No important change in the animals' body weight was showed after the treatment **(F)**.

6.2.2 BITC ameliorates the morphological aspects of ATC studied by Masson's Trichrome Staining

The morphology of orthotopic 8305C tumors was observed on histologic sections after Masson's trichrome staining. Overall, the presence of collagen fibers stained in blue around the tumors, as well as their invasion within the tumors are noticeable in the ATC group compared to sham group. Our data showed that BITC treatments at doses of 30 and 100 mg/kg were able to ameliorate these morphological aspects as showed in Figure 6.

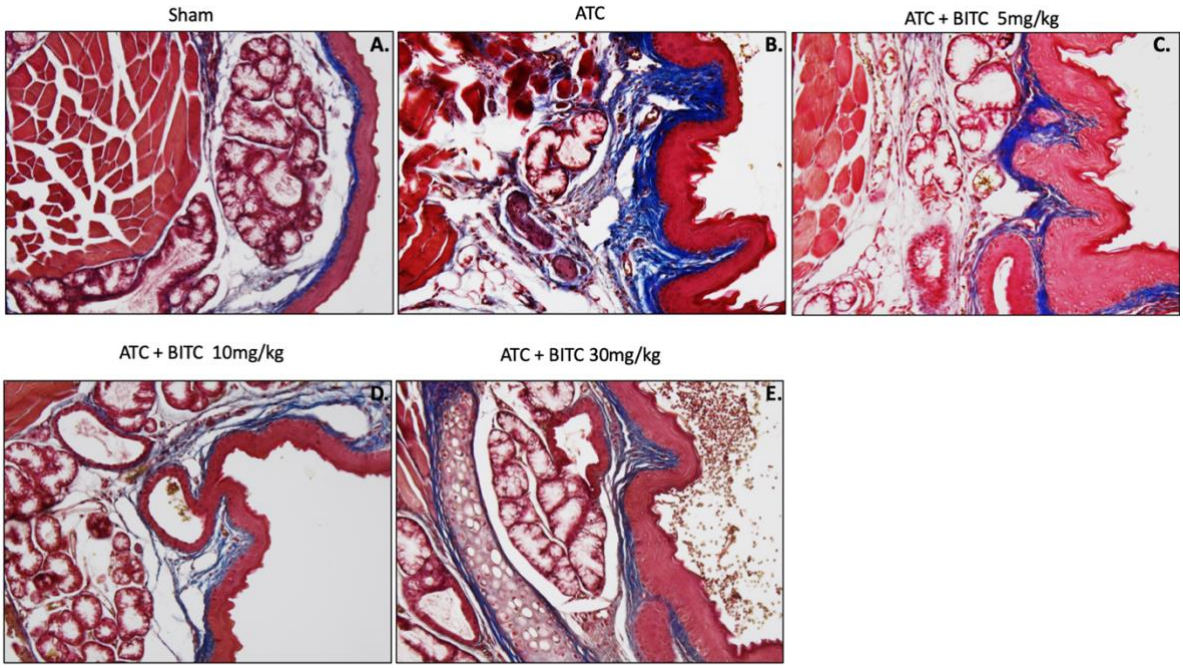

Figure 6. Effect of BITC on tumor morphology in an orthotopic model of ATC. Masson's trichrome staining of ATC (8305C) tumors developed in nude mice orthotopically; cytoplasm is stained in red; the collagen is stained in blue.

6.2.3 BITC modulates EMT markers expression on ATC orthotopic tumors

EMT causes significant morphological changes from the epithelial to mesenchymal phenotype and promotes greater progression and invasion [171]. In this context, our results showed a significant upregulation of mesenchymal marker N-cadherin and a downregulation of epithelial factor E-cadherin in the ATC group compared to sham group (Figure 7.B-H). BITC

treatments at doses of 30 and 100 mg/kg led to significant downregulation of N-cadherin expression and upregulation of E-cadherin expression in orthotopic tumors as showed in Figure 7, carrying out an important role in reducing tumoral migration and invasion.

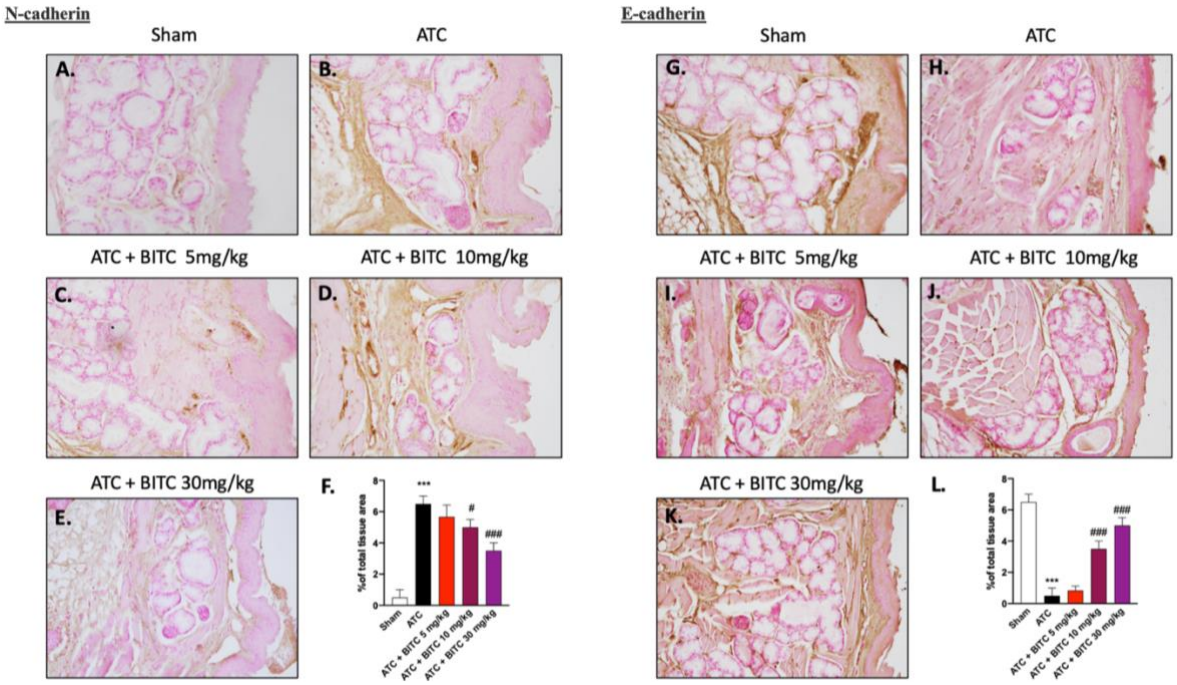

Figure 7. Effect of BITC on EMT markers in an orthotopic model of ATC. The immunohistochemical assay revealed a significant increase of N-cadherin expression in the ATC group **(B)** compared to Sham mice **(A)**; this expression was reduced following treatment with BITC at doses of 10 mg/kg **(D)** and 30 mg/kg **(E)** compared to ATC group **(B)**. At the same time the staining revelated a significant reduction of E-Cadherin expression in the ATC group **(H)**, compared to Sham mice **(G);** 10 mg/kg **(J)** and 30 mg/kg **(K)** BITC treatments caused an important increase of E-Cadherin expression compared to ATC group **(H)**. The data are representative of at least three independent experiments. The sections were observed and photographed at 20× magnification. *** $p < 0.001$ vs Sham; # $p < 0.05$ vs. ATC; ## $p < 0.01$ vs ATC; ### $p < 0.001$ vs ATC.

6.2.4 BITC reduces S100 expression on ATC orthotopic tumors

S100A4 protein is also known as "metastasin", and its increased expression has been associated with many malignancies including thyroid cancer [172-175]. Expression of S100A4 is increased in ATC group compared to sham counterpart where expression is low or non-existent. Expression of S100A4 has been shown to be inversely related to E-cadherin expression in some cancers, leading to a more aggressive phenotype and hence a worse prognosis. BITC treatments mainly at doses of 30 mg/kg and 100 mg/kg were able to reduce S100 expression compared to the ATC group as showed in Figure 8.

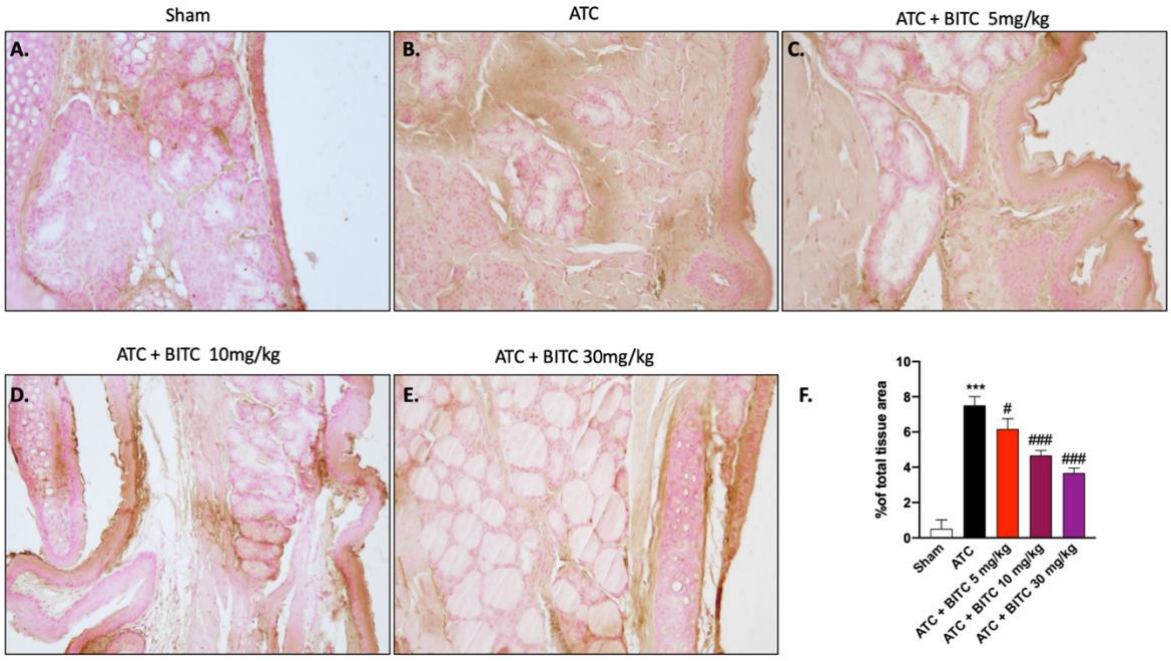

Figure 8. Effect of BITC on S100 expression in an orthotopic model of ATC. The immunohistochemical assay revealed a significant increase of S100 expression in the ATC group **(B)** compared to Sham mice **(A)**; this expression was reduced following treatment with BITC at doses of 10 mg/kg **(D)** and 30 mg/kg **(E)** compared to ATC group **(B)**. The data are representative of at least three independent experiments. The sections were observed and photographed at 20× magnification. *** $p < 0.001$ vs Sham; # $p < 0.05$ vs. ATC; ### $p < 0.001$ vs ATC.

6.2.5 BITC confirms the modulation of apoptotic and autophagy pathways in the orthotopic model

To confirm the key role of apoptosis in the progression of ATC we evaluated some of the main apoptotic factors by western blot analysis, also on the thyroid samples collected in the ATC orthotopic model. The results showed that BITC was able to significantly increase BID and BAD expression and reduce BCL-2 expression, mainly at the highest doses as showed on Figure 10. For the same reason we also investigated the expression of autophagy markers, demonstrating that BITC was able to significantly increase the expression of PTEN, and reduce the expression of p-mTOR, p62 and SOS-1 mainly at doses of 30 and 100 mg/kg compared to ATC group, as showed in Figure 9.

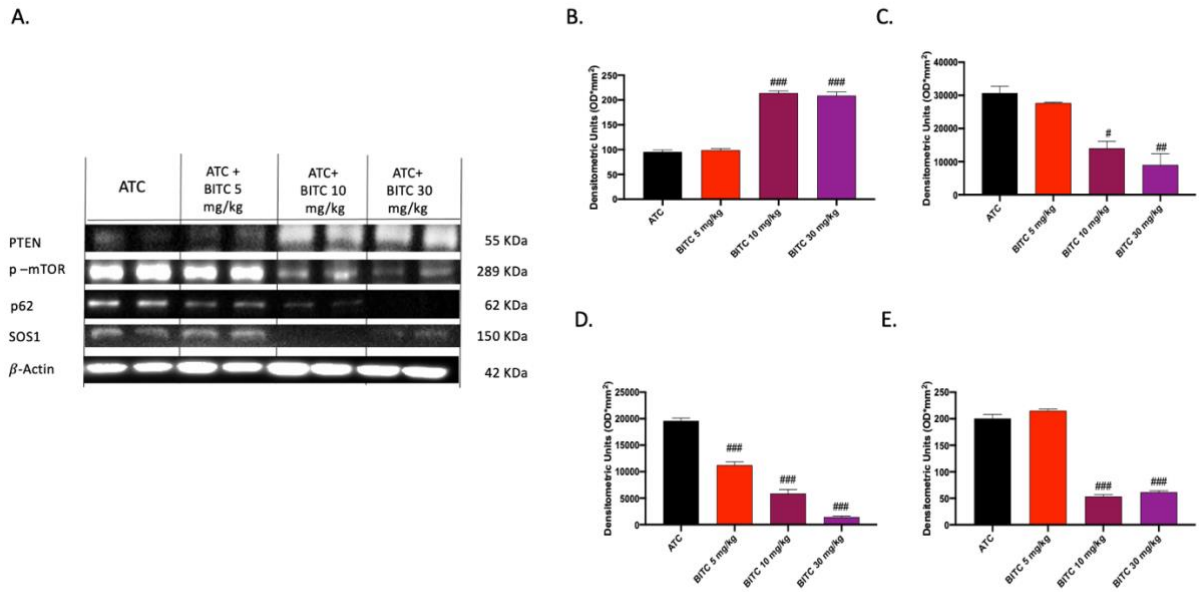

Figure 9. Effect of BITC on autophagy markers expression in an orthotopic model of ATC. The blots revealed a significant modulation of autophagy markers expression following BITC treatment **(A)**. BITC at the doses of 10 mg/kg and 30 mg/kg was able to significantly

increase the expression of PTEN **(B),** and reduce p-mTOR **(C),** p62 **(D)** and SOS-1**(E**) proteins**;** # p < 0.05 vs ATC; ## p < 0.01 vs ATC; ### p < 0.001 vs ATC.

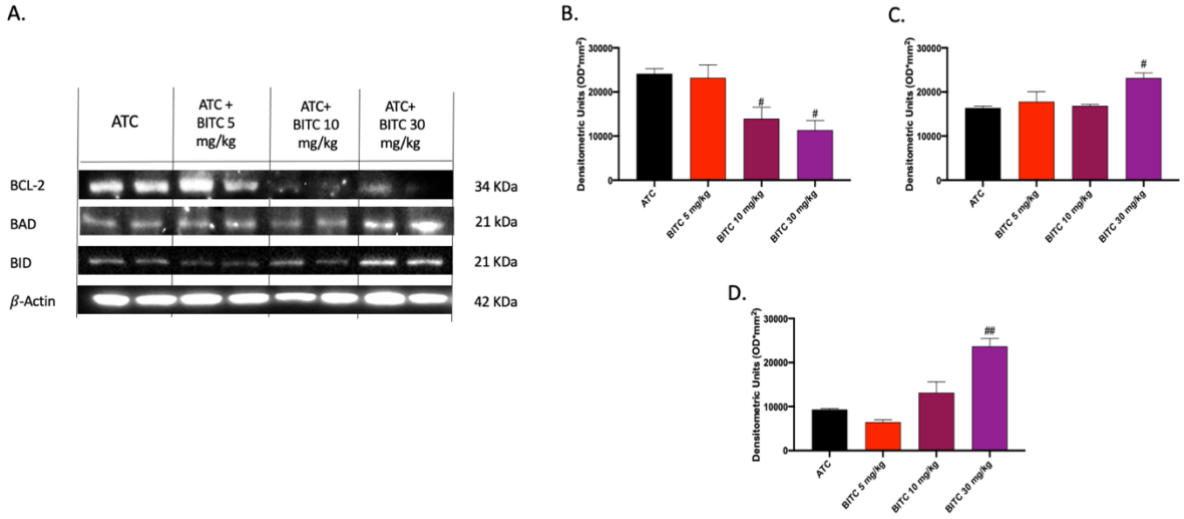

Figure 10. Effect of BITC on apoptosis markers expression in an orthotopic model of ATC. The blots revealed a significant modulation of apoptosis markers expression following BITC treatment **(A)**. BITC at the doses of 10 mg/kg and 30 mg/kg was able to significantly reduce the expression of anti-apoptotic protein BCL-2 **(B)** # p < 0.05 vs ATC**;** at the same time 30 mg/kg BITC treatment increased the expression of pro-apoptotic BAD **(C),** and BID **(D)** markers compared to ATC group; # p < 0.05 vs ATC; ## p < 0.01 vs ATC.

6.3 Experiments conducted at the Institute of Oncology Research (IOR), Bellinzona, Switzerland

6.3.1 Effect of Palbociclib and Cisplatin on 8305C cells proliferation

To evaluate the effect of Palbociclib and Cisplatin on 8305C cell proliferation we used Incucyte imaging analysis. After 84 hrs of treatment with Palbociclib at different

concentrations (20 µM, 10 µM, 5 µM, 2.5 µM, 1.2 µM, 0.6 µM, 0.3 µM and 0.15 µM) and Cisplatin (50 µM, 25 µM, 12 µM, 6 µM, 3 µM, 1.5 µM, 0.7 µM and 0.3 µM) our results show that Palbociclib and Cisplatin treatments were able to decrease cells proliferation in a significant manner compared to vehicle at all of concentrations as showed in Figure 11 (A, B). Based on the Proliferation assay results, we decided to investigate in further analysis only Palbociclib at the concentration of 5 µM and Cisplatin 5 µM and 1 µM because they represent the most effective concentrations. To confirm the efficacy of these concentrations we also performed a Crystal Violet assay. Our result confirmed that these drugs were able to significantly reduce cell proliferation compared to untreated 8305C cells as showed in Figure 11 (C).

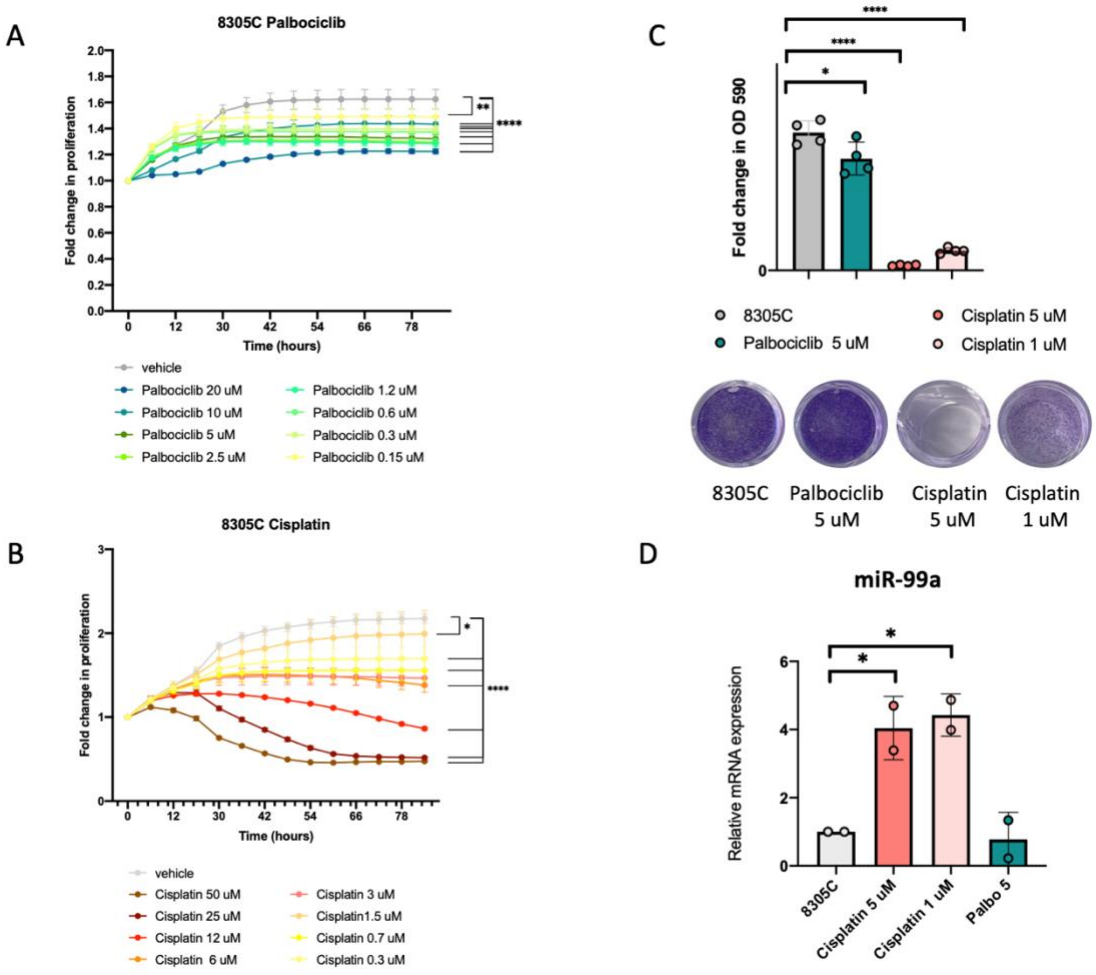

Figure 11. Effect of Cisplatin and Palbociclib on 8305C proliferation and miR-99a expression. Titration of Palbociclib (A) and Cisplatin (B) concentrations after 84 hrs of treatment in 8305C cells, by Incucyte S3 in vitro system (Essenbioscience). Crystal violet assay to confirm the antiproliferative effect of Cisplatin 5 μM and 1 μM and Palbocicliclib 5 μM in 8305C cells (C). qRT-PCR for miR-99a on 8305C cells treated with Cisplatin 5 μM and 1 μM and Palbociclib 5 μM (D). * $p < 0.05$ vs vehicle; ** $p< 0.01$; **** $p < 0.0001$ vs vehicle.

6.3.2 Effect of Cisplatin and Palbociclib on miR-99a modulation

Recent evidence suggests that BITC acts through overexpression of the tumor suppressor miR-99a. After confirming the expression of miR-99a by RT-PCR in 8305C cells, we evaluated how treatments with Palbociclib, Cisplatin and BITC may be able to modulate the expression of this microRNA. Our results showed that Cisplatin 1 μM and 5 μM were able to significantly increase the expression of miR-99a compared to untreated cell, while Palbociclib 5 μM did not result in significant overexpression (Figure 11 D). The results about BITC are ongoing, but it would be interesting to understand if BITC has a greater effect compared to these drugs in miR-99a modulation, as it would confirm what is present in the literature regarding a possible mechanism of action.

6.3.3 Evaluation of involvement of senescence response on 8305C cell line

Senescent cells undergo characteristic changes, including chromatin rearrangement, cell cycle arrest and increased senescence-associated β-galactosidase (SA-β-gal) activity [140]. For this reason, we evaluated how treatments with Palbociclib, Cisplatin and BITC may be able to modulate the senescence response in 8305C cell line. Our result showed that Cisplatin 5 μM and 1 μM and Palbociclib 5 μM were able to significantly increase the activity of SA-β-gal

compared to untreated cells (Figura 12 A). This senescent effect in Cisplatin 5 μM and 1 μM appears to be mediated by mRNA overexpression of senescence-associated markers p16 and p21, whereas p27 does not appear to be significantly involved in inducing stable cell cycle arrest (Figure 12 C, D,E).

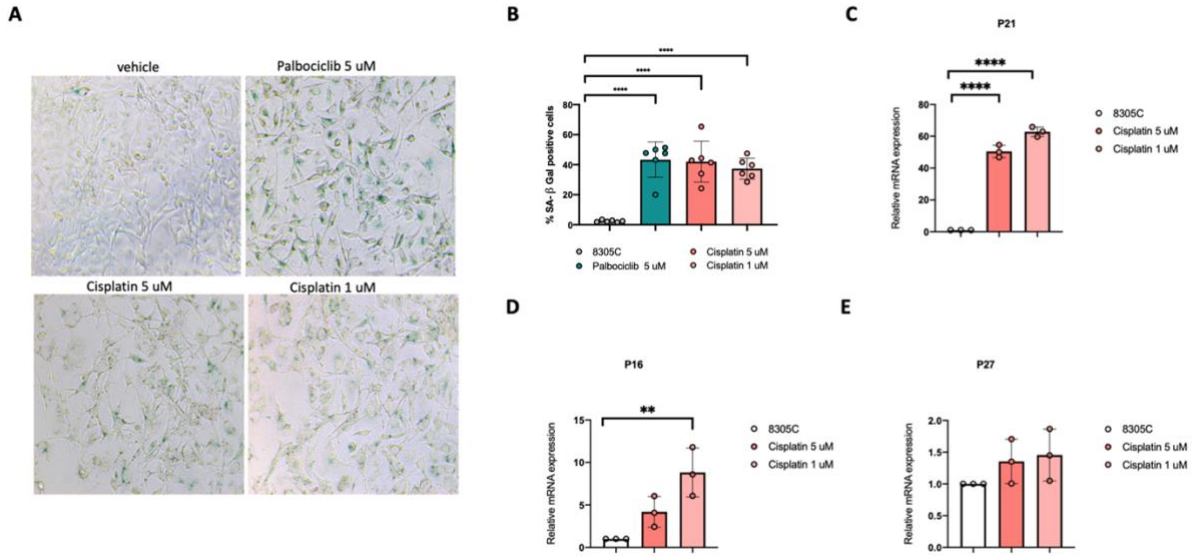

Figure 12. Effect of Cisplatin, Palbociclib on induction of senescence response in 8305C cells. SA-β Gal assay on 8305C cells treated with Cisplatin 5 μM and 1 μM and Palbocicliclib 5 μM (A) and respective quantification (B). qRT-PCR for p21 (C), p16 (D) and p27 (E) senescence markers on 8305C cells treated with Cisplatin 5 μM and 1 μM and Palbociclib 5 μM. ** $p < 0.01$; **** $p < 0.0001$ vs vehicle.

6.3.4 Effect of Cisplatin and Palbociclib in the induction of Senescence-Associated Secretory Phenotype (SASP) on 8305C cell line

Features of senescence is also the secretion of pro-inflammatory cytokines, growth factors and matrix metalloproteinases (MMPs), collectively termed the senescence-associated secretory phenotype (SASP), which is primarily responsible for the paracrine effects of senescent cells,

by reinforcing the senescent growth arrest. In line with our previous findings, conditioned media (c.m.) from senescent 8305C cells treated with Cisplatin 1 μM and Palbociclib 5 μM manteined the prosenescence effect and arrested the proliferation of 8305C parental cells (Figure 13). The results about BITC are ongoing, but it would be interesting to understand if BITC has an effect in the modulation of senescence response, in order to consider its use as a possible pro-senescence therapy.

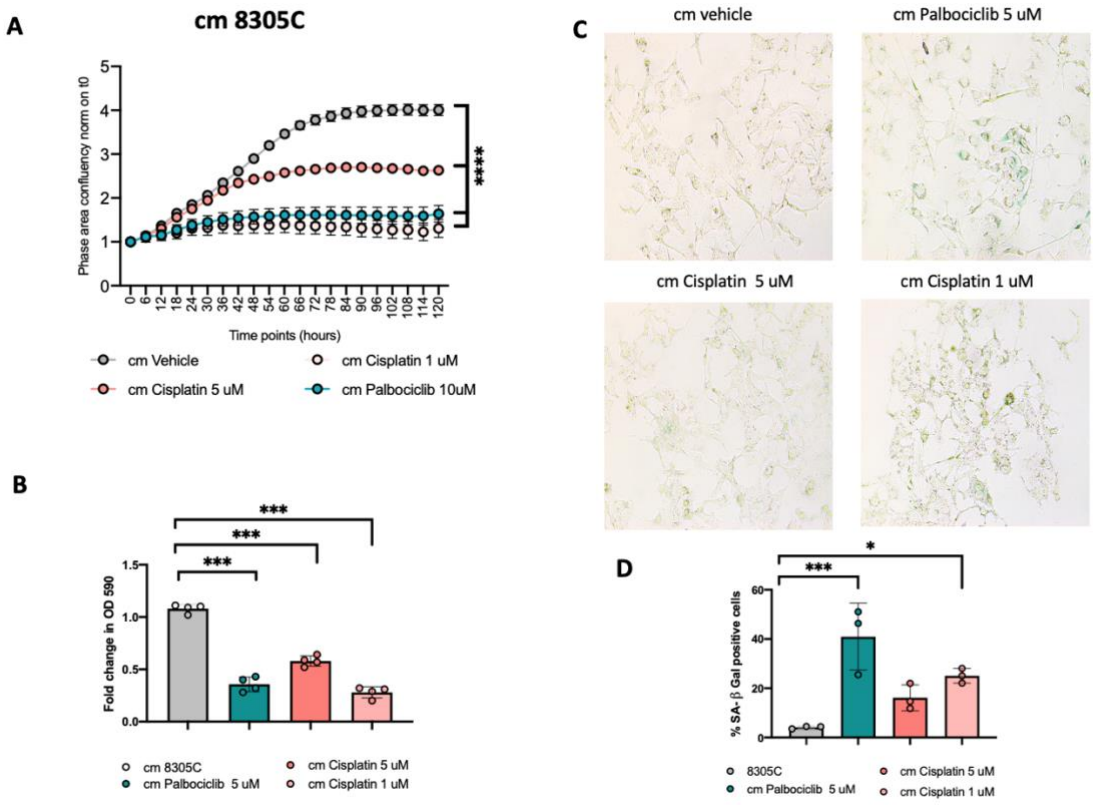

Figure 13. Paracrine effect of Cisplatin, Palbociclib on induction of senescence response in 8305C cells. Proliferation assay (A) and Crystal Violet assay (B) on parental 8305C cells treated with conditioned media (cm) from senescent 8305C cells treated with Cisplatin 5 μM and 1 μM and Palbocicicliib 5 μM. SA-β Gal assay on 8305C cells treated with cm of 8305C cells treated with Cisplatin 5 μM and 1 μM and Palbocicicliib 5 μM (C) and respective quantification (D). * $p< 0.05$; *** $p< 0.001$; **** $p < 0.0001$ vs vehicle.

CHAPTER SEVEN: DISCUSSION

7. Discussion

Although ATC is the type of thyroid cancer with the lowest incidence, compared to differentiated carcinomas, it is characterized by a high risk of recurrence and poor prognosis [176]. Conventional treatments including surgery and chemotherapy have proven ineffective for ATC and have failed to improve survival rates [177]. Accumulating evidence suggests that autophagy plays a key role in thyroid cancer, acting to promote tumor cell viability and metastatic disease through maintenance of cancer stem cells (CSCs), supporting epithelial-to-mesenchymal transition (EMT), and preventing tumor cell death [178]. Autophagy refers to the process of maintaining cellular homeostasis by which dysfunctional cellular components, such as damaged proteins or aging organelles, are recruited directly into lysosomes via autophagic vacuoles for degradation [179]. Several signaling pathways participate in autophagy formation, including PI3K/Akt/mTOR pathways, MAPK, and AMP-activated protein kinase (AMPK), with PI3K/Akt/mTOR signaling reported more. In the PI3K-AKT-mTOR pathway, mutations of PIK3CA, PTEN and AKT1 were detected in patients with anaplastic thyroid carcinoma. PTEN (Phosphatase and tensin homolog) encoded PTEN enzyme, which acts as a tumor suppressor by regulating cell division and preventing abnormal cell growth and division [180]. mTOR is a regulatory protein, involved in the PI3K/Akt/mTOR pathway, which facilitates iodine uptake by thyroid cells, resulting in the promotion of cell proliferation and survival. Genetic alterations occurring in this pathway play a role in the progression of ATC. mTOR is a highly attractive target via several therapeutic strategies [181-183]. mTOR interacts with the adaptor protein p62 (also known as sequestosome-1, SQSTM-1 or A170) in the formation of the complex in the lysosomal compartment. P62 is a crucial molecule in the regulation of cell growth, survival and proliferation. P62 recognizes polyubiquitin chains through its C-terminal domain and binds to LC3, thus promoting autophagic degradation of ubiquitinated loads. P62 colocalized with LAMP2 (a lysosomal

marker) and Rags and the translocation of the mTORC1 complex to the lysosomal surface is necessary for the interaction of mTOR [184]. Another process that can indirectly influence autophagy is the activation of the Ras-MAPK pathway, mediated by SOS1 (Son of Sevenless Homolog 1). SOS1 participates in the transduction of signals from growth factor receptors and may respond to stressors such as oxidative stress or DNA damage, which can trigger autophagic responses as a means of removing damaged cellular components and promoting cell survival. Activating Ras can, in fact, activate the mTOR path, which is a key negative regulator of autophagy [185]. Our results showed that BITC was able to increase the expression of the tumor suppressor gene PTEN, leading to the modulation of the autophagic pathway mediated by downregulation of p-mTOR, P62 and SOS1 and upregulation of LC3 and LAMP2, contributing to the improvement of apoptosis and repression of ATC cell proliferation and invasion. The activation of apoptotic processes has been confirmed by our data obtained both *in vitro* and *in vivo* through the increase in the expression of proapoptotic markers such as BID, BAX and BAD and the decrease of antiapoptotic factors such as BCL-2. The epithelial-mesenchymal transition (EMT) is a distinctive process of metastatic disease, characterized by the loss of cell-to-cell adhesion capabilities and the consequent transition to a mesenchymal phenotype, which results in increased cell plasticity and motility, as well as resistance to apoptosis [178]. The loss of E-cadherin leads to activation of the Wnt/β-catenin pathway and subsequent transcription of genes that promote proliferation and migration. Our results showed that BITC treatments were able to reduce the migration and invasiveness both *in vitro* and *in vivo* and to promote the expression of E-cadherin and the loss of genes typically expressed in mesenchymal cells, like N-cadherin and S100. Growing evidence suggests that the immune response may influence tumorigenesis and thyroid cancer progression [169,186]. Networks of cytokines and chemokines present in the tumor microenvironment appear to be involved in the development of the immune response may influence the progression of thyroid cancer. IL-17 is a cytokine produced by a subset of lymphocytes known as Th17 cells and

shows an important function in inducing the production of high concentrations of IL-1β, TNF-α, TGF-β, chemokine and matrix metalloproteinase mediators that are widely present in the tumor microenvironment [187]. Tumor necrosis factor receptor (TNFR)-associated factors 6 (TRAF6) is part of the heterotrimeric complex required for IL-17 signaling and gene expression [188] and is implicated in the downregulation of the NFκB pathway while promoting the p38 MAPK pathway. In this work we demonstrated that BITC treatment was able to reduce IL-17, IL-12p70 levels and TRAF6 expression and to increase pP38, suggesting a key role also in the modification of the immune response and in the reduction of inflammatory cytokine levels in the tumor microenvironment.

During the third year of my PhD, I had the opportunity to be hosted at the Institute of Oncology Research (IOR) in Bellinzona, Switzerland, for a period of six months, during which I deepened my knowledge and skills related to the mechanisms underlying cellular oncology. The work in collaboration with the IOR stems from the idea of linking the research topics carried out at the Molecular Oncology laboratory of Professor Andrea Alimonti with the studies previously conducted for my PhD project. This laboratory, in fact, is highly specialized in the identification and development of pro-senescence and senolytic compounds for the treatment of prostate cancer. Cellular senescence is a condition of quiescence between life and death that cells can be subjected to in response to different internal and external stimuli, such as dysfunctional telomeres, mitogenic signals, oxidative stress, oncogene activation, ionizing radiation, and treatment with anticancer therapies, which can cause persistent DNA damage and trigger permanent cell cycle arrest [139,140]. This stable cell cycle arrest correlates with increasing level of cell cycle inhibitors, including p16INK4a, p21CIP1, and p27, and limits the proliferative lifespan of the cell. Therefore, due to its antiproliferative effects, cellular senescence is currently being recognized as a potent antitumor mechanism, paving the way for new treatments for cancer therapy [142]. Some isothiocyanates, including Phenethyl

isothiocyanate (PEITC) and sulforaphane (SFN) have been shown in preclinical studies to have a prosescence action in different types of tumors [145-149], but to date there is no evidence regarding the effect of BITC in modulating this senescent response. Therefore, these evidences have provided the rationale to continue the studies related to this compound, which aim to investigate the involvement of cellular senescence as a mechanism below the antitumor action of BITC. The hypothesis underlying this study is that the over-expression of miR-99a induced by BITC may be linked in some way to the induction of a senescent response and consequently to the reduction of tumor progression, providing the necessary elements for the identification of a new therapeutic strategy. For this reason, some experiments were conducted in order to evaluate how the expression of miR-99a changes in relation to the activation of cellular senescence induced by some known pro-senescence stimuli such as Palbociclib and Cisplatin. Our results showed that Cisplatin and Palbociclib were able to induce a significant autocrine and paracrine senescent response in ATC cell line, demonstrated by increased SA-β-gal activity and mRNA overexpression of senescence-associated markers p16 and p21. At the same time, Cisplatin has been shown to increase the expression of the tumor suppressor miR-99a, suggesting the possible existence of a link between the upregulation of this small non-coding mRNA and the activation of cellular senescence.

CHAPTER EIGHT: CONCLUSIONS

8. Conclusions

The results suggest that treatment with BITC has the potential to influence various cellular processes like autophagy, apoptosis, EMT (Epithelial-Mesenchymal Transition) and inflammation in thyroid cancer. This indicates that BITC may play a role in influencing the development of thyroid cancer by modulating several signaling pathways often dysregulated and correlated with the initiation and progression of anaplastic thyroid carcinoma. Ongoing studies aim to investigate whether this antitumor effect of BITC can be mediated by an increase in miR-99a expression directly linked to the activation of a pro-senescence response. Although further studies are required to confirm these initial findings especially in human clinical trials, to better understand its safety and effectiveness in cancer prevention and treatment, BITC could be explored as a potential therapeutic approach for managing thyroid cancer.

CHAPTER NINE: REFERENCES

9. *References*

1. Baloch, Z.W.; Asa, S.L.; Barletta, J.A.; Ghossein, R.A.; Juhlin, C.C.; Jung, C.K.; LiVolsi, V.A.; Papotti, M.G.; Sobrinho-Simoes, M.; Tallini, G.; et al. Overview of the 2022 WHO Classification of Thyroid Neoplasms. *Endocr Pathol* **2022**, *33*, 27-63, doi:10.1007/s12022-022-09707-3.

2. Asa, S.L. The Current Histologic Classification of Thyroid Cancer. *Endocrinol Metab Clin North Am* **2019**, *48*, 1-22, doi:10.1016/j.ecl.2018.10.001.

3. Abe, I.; Lam, A.K. Anaplastic thyroid carcinoma: Updates on WHO classification, clinicopathological features and staging. *Histol Histopathol* **2021**, *36*, 239-248, doi:10.14670/HH-18-277.

4. Force, U.S.P.S.T.; Bibbins-Domingo, K.; Grossman, D.C.; Curry, S.J.; Barry, M.J.; Davidson, K.W.; Doubeni, C.A.; Epling, J.W., Jr.; Kemper, A.R.; Krist, A.H.; et al. Screening for Thyroid Cancer: US Preventive Services Task Force Recommendation Statement. *JAMA* **2017**, *317*, 1882-1887, doi:10.1001/jama.2017.4011.

5. Haddad, R.I.; Bischoff, L.; Ball, D.; Bernet, V.; Blomain, E.; Busaidy, N.L.; Campbell, M.; Dickson, P.; Duh, Q.Y.; Ehya, H.; et al. Thyroid Carcinoma, Version 2.2022, NCCN Clinical Practice Guidelines in Oncology. *J Natl Compr Canc Netw* **2022**, *20*, 925-951, doi:10.6004/jnccn.2022.0040.

6. DeGroot, L.J.; Zhang, R. Clinical review 131: Gene therapy for thyroid cancer: where do we stand? *J Clin Endocrinol Metab* **2001**, *86*, 2923-2928, doi:10.1210/jcem.86.7.7653.

7. Zhang, Y.; Xing, Z.; Liu, T.; Tang, M.; Mi, L.; Zhu, J.; Wu, W.; Wei, T. Targeted therapy and drug resistance in thyroid cancer. *Eur J Med Chem* **2022**, *238*, 114500, doi:10.1016/j.ejmech.2022.114500.

8. Laha, D.; Nilubol, N.; Boufraqech, M. New Therapies for Advanced Thyroid Cancer. *Front Endocrinol (Lausanne)* **2020**, *11*, 82, doi:10.3389/fendo.2020.00082.

9. Kim, E.J.; Eom, S.J.; Hong, J.E.; Lee, J.Y.; Choi, M.S.; Park, J.H. Benzyl isothiocyanate inhibits basal and hepatocyte growth factor-stimulated migration of breast cancer cells. *Mol Cell Biochem* **2012**, *359*, 431-440, doi:10.1007/s11010-011-1039-3.

10. Po, W.W.; Choi, W.S.; Khing, T.M.; Lee, J.Y.; Lee, J.H.; Bang, J.S.; Min, Y.S.; Jeong, J.H.; Sohn, U.D. Benzyl Isothiocyanate-Induced Cytotoxicity via the Inhibition of Autophagy and Lysosomal Function in AGS Cells. *Biomol Ther (Seoul)* **2022**, *30*, 348-359, doi:10.4062/biomolther.2022.019.

11. Lin, J.F.; Tsai, T.F.; Yang, S.C.; Lin, Y.C.; Chen, H.E.; Chou, K.Y.; Hwang, T.I. Benzyl isothiocyanate induces reactive oxygen species-initiated autophagy and apoptosis in human prostate cancer cells. *Oncotarget* **2017**, *8*, 20220-20234, doi:10.18632/oncotarget.15643.

12. Sehrawat, A.; Singh, S.V. Benzyl isothiocyanate inhibits epithelial-mesenchymal transition in cultured and xenografted human breast cancer cells. *Cancer Prev Res (Phila)* **2011**, *4*, 1107-1117, doi:10.1158/1940-6207.CAPR-10-0306.

13. Lin, J.F.; Tsai, T.F.; Lin, Y.C.; Chen, H.E.; Chou, K.Y.; Hwang, T.I. Benzyl isothiocyanate suppresses IGF1R, FGFR3 and mTOR expression by upregulation of miR-99a-5p in human bladder cancer cells. *Int J Oncol* **2019**, *54*, 2106-2116, doi:10.3892/ijo.2019.4763.

14. Zhang, Q.C.; Pan, Z.H.; Liu, B.N.; Meng, Z.W.; Wu, X.; Zhou, Q.H.; Xu, K. Benzyl isothiocyanate induces protective autophagy in human lung cancer cells through an endoplasmic reticulum stress-mediated mechanism. *Acta Pharmacol Sin* **2017**, *38*, 539-550, doi:10.1038/aps.2016.146.

15. Liu, X.; Abe-Kanoh, N.; Liu, Y.; Zhu, B.; Munemasa, S.; Nakamura, T.; Murata, Y.; Nakamura, Y. Inhibition of phosphatidylinositide 3-kinase impairs the benzyl isothiocyanate-induced accumulation of autophagic molecules and Nrf2 in human colon cancer cells. *Biosci Biotechnol Biochem* **2017**, *81*, 2212-2215, doi:10.1080/09168451.2017.1374830.

16. Xie, B.; Nagalingam, A.; Kuppusamy, P.; Muniraj, N.; Langford, P.; Gyorffy, B.; Saxena, N.K.; Sharma, D. Benzyl Isothiocyanate potentiates p53 signaling and antitumor effects against breast cancer through activation of p53-LKB1 and p73-LKB1 axes. *Sci Rep* **2017**, *7*, 40070, doi:10.1038/srep40070.

17. Cho, H.J.; Lim, D.Y.; Kwon, G.T.; Kim, J.H.; Huang, Z.; Song, H.; Oh, Y.S.; Kang, Y.H.; Lee, K.W.; Dong, Z.; et al. Benzyl Isothiocyanate Inhibits Prostate Cancer Development in the Transgenic Adenocarcinoma Mouse Prostate (TRAMP) Model, Which Is Associated with the Induction of Cell Cycle G1 Arrest. *Int J Mol Sci* **2016**, *17*, 264, doi:10.3390/ijms17020264.

18. Lai, K.C.; Huang, A.C.; Hsu, S.C.; Kuo, C.L.; Yang, J.S.; Wu, S.H.; Chung, J.G. Benzyl isothiocyanate (BITC) inhibits migration and invasion of human colon cancer HT29 cells by inhibiting matrix metalloproteinase-2/-9 and urokinase plasminogen (uPA) through PKC and MAPK signaling pathway. *J Agric Food Chem* **2010**, *58*, 2935-2942, doi:10.1021/jf9036694.

19. Lin, J.F.; Tsai, T.F.; Liao, P.C.; Lin, Y.H.; Lin, Y.C.; Chen, H.E.; Chou, K.Y.; Hwang, T.I. Benzyl isothiocyanate induces protective autophagy in human prostate cancer cells via inhibition of mTOR signaling. *Carcinogenesis* **2013**, *34*, 406-414, doi:10.1093/carcin/bgs359.

20. Hu, J.; Yuan, I.J.; Mirshahidi, S.; Simental, A.; Lee, S.C.; Yuan, X. Thyroid Carcinoma: Phenotypic Features, Underlying Biology and Potential Relevance for Targeting Therapy. *Int J Mol Sci* **2021**, *22*, doi:10.3390/ijms22041950.

21. Favus, M.J.; Schneider, A.B.; Stachura, M.E.; Arnold, J.E.; Ryo, U.Y.; Pinsky, S.M.; Colman, M.; Arnold, M.J.; Frohman, L.A. Thyroid cancer occurring as a late consequence of head-and-neck irradiation. Evaluation of 1056 patients. *N Engl J Med* **1976**, *294*, 1019-1025, doi:10.1056/NEJM197605062941901.

22. Furukawa, K.; Preston, D.; Funamoto, S.; Yonehara, S.; Ito, M.; Tokuoka, S.; Sugiyama, H.; Soda, M.; Ozasa, K.; Mabuchi, K. Long-term trend of thyroid cancer risk

among Japanese atomic-bomb survivors: 60 years after exposure. *Int J Cancer* **2013**, *132*, 1222-1226, doi:10.1002/ijc.27749.

23. Schonfeld, S.J.; Lee, C.; Berrington de Gonzalez, A. Medical exposure to radiation and thyroid cancer. *Clin Oncol (R Coll Radiol)* **2011**, *23*, 244-250, doi:10.1016/j.clon.2011.01.159.

24. Knobel, M.; Medeiros-Neto, G. Relevance of iodine intake as a reputed predisposing factor for thyroid cancer. *Arq Bras Endocrinol Metabol* **2007**, *51*, 701-712, doi:10.1590/s0004-27302007000500007.

25. Horn-Ross, P.L.; Morris, J.S.; Lee, M.; West, D.W.; Whittemore, A.S.; McDougall, I.R.; Nowels, K.; Stewart, S.L.; Spate, V.L.; Shiau, A.C.; et al. Iodine and thyroid cancer risk among women in a multiethnic population: the Bay Area Thyroid Cancer Study. *Cancer Epidemiol Biomarkers Prev* **2001**, *10*, 979-985.

26. Belfiore, A.; La Rosa, G.L.; La Porta, G.A.; Giuffrida, D.; Milazzo, G.; Lupo, L.; Regalbuto, C.; Vigneri, R. Cancer risk in patients with cold thyroid nodules: relevance of iodine intake, sex, age, and multinodularity. *Am J Med* **1992**, *93*, 363-369, doi:10.1016/0002-9343(92)90164-7.

27. Chen, G.G.; Vlantis, A.C.; Zeng, Q.; van Hasselt, C.A. Regulation of cell growth by estrogen signaling and potential targets in thyroid cancer. *Curr Cancer Drug Targets* **2008**, *8*, 367-377, doi:10.2174/156800908785133150.

28. Wiench, M.; Wloch, J.; Oczko, M.; Gubala, E.; Jarzab, B. [Rearrangement of the RET gene in papillary thyroid carcinoma]. *Wiad Lek* **2001**, *54 Suppl 1*, 64-71.

29. Xing, M. BRAF mutation in thyroid cancer. *Endocr Relat Cancer* **2005**, *12*, 245-262, doi:10.1677/erc.1.0978.

30. Nozhat, Z.; Hedayati, M. PI3K/AKT Pathway and Its Mediators in Thyroid Carcinomas. *Mol Diagn Ther* **2016**, *20*, 13-26, doi:10.1007/s40291-015-0175-y.

31. Xu, B.; Ghossein, R. Genomic Landscape of poorly Differentiated and Anaplastic Thyroid Carcinoma. *Endocr Pathol* **2016**, *27*, 205-212, doi:10.1007/s12022-016-9445-4.

32. Landa, I.; Ibrahimpasic, T.; Boucai, L.; Sinha, R.; Knauf, J.A.; Shah, R.H.; Dogan, S.; Ricarte-Filho, J.C.; Krishnamoorthy, G.P.; Xu, B.; et al. Genomic and transcriptomic hallmarks of poorly differentiated and anaplastic thyroid cancers. *J Clin Invest* **2016**, *126*, 1052-1066, doi:10.1172/JCI85271.

33. Jeon, M.J.; Chun, S.M.; Kim, D.; Kwon, H.; Jang, E.K.; Kim, T.Y.; Kim, W.B.; Shong, Y.K.; Jang, S.J.; Song, D.E.; et al. Genomic Alterations of Anaplastic Thyroid Carcinoma Detected by Targeted Massive Parallel Sequencing in a BRAF(V600E) Mutation-Prevalent Area. *Thyroid* **2016**, *26*, 683-690, doi:10.1089/thy.2015.0506.

34. Kunstman, J.W.; Juhlin, C.C.; Goh, G.; Brown, T.C.; Stenman, A.; Healy, J.M.; Rubinstein, J.C.; Choi, M.; Kiss, N.; Nelson-Williams, C.; et al. Characterization of the mutational landscape of anaplastic thyroid cancer via whole-exome sequencing. *Hum Mol Genet* **2015**, *24*, 2318-2329, doi:10.1093/hmg/ddu749.

35. Cradic, K.W.; Milosevic, D.; Rosenberg, A.M.; Erickson, L.A.; McIver, B.; Grebe, S.K. Mutant BRAF(T1799A) can be detected in the blood of papillary thyroid carcinoma patients and correlates with disease status. *J Clin Endocrinol Metab* **2009**, *94*, 5001-5009, doi:10.1210/jc.2009-1349.

36. Fagin, J.A.; Matsuo, K.; Karmakar, A.; Chen, D.L.; Tang, S.H.; Koeffler, H.P. High prevalence of mutations of the p53 gene in poorly differentiated human thyroid carcinomas. *J Clin Invest* **1993**, *91*, 179-184, doi:10.1172/JCI116168.

37. Eberhardt, N.L.; Grebe, S.K.; McIver, B.; Reddi, H.V. The role of the PAX8/PPARgamma fusion oncogene in the pathogenesis of follicular thyroid cancer. *Mol Cell Endocrinol* **2010**, *321*, 50-56, doi:10.1016/j.mce.2009.10.013.

38. Liu, X.; Bishop, J.; Shan, Y.; Pai, S.; Liu, D.; Murugan, A.K.; Sun, H.; El-Naggar, A.K.; Xing, M. Highly prevalent TERT promoter mutations in aggressive thyroid cancers. *Endocr Relat Cancer* **2013**, *20*, 603-610, doi:10.1530/ERC-13-0210.

39. Landa, I.; Ganly, I.; Chan, T.A.; Mitsutake, N.; Matsuse, M.; Ibrahimpasic, T.; Ghossein, R.A.; Fagin, J.A. Frequent somatic TERT promoter mutations in thyroid cancer: higher prevalence in advanced forms of the disease. *J Clin Endocrinol Metab* **2013**, *98*, E1562-1566, doi:10.1210/jc.2013-2383.

40. Cancer, I.A.f.R.o. WHO Classification of Tumours Editorial Board: Endocrine and Neuroendocrine Tumours. **2022**.

41. Mete, O.; Asa, S.L. Pitfalls in the diagnosis of follicular epithelial proliferations of the thyroid. *Adv Anat Pathol* **2012**, *19*, 363-373, doi:10.1097/PAP.0b013e318271a5ac.

42. Parma, J.; Duprez, L.; Van Sande, J.; Cochaux, P.; Gervy, C.; Mockel, J.; Dumont, J.; Vassart, G. Somatic mutations in the thyrotropin receptor gene cause hyperfunctioning thyroid adenomas. *Nature* **1993**, *365*, 649-651, doi:10.1038/365649a0.

43. Gozu, H.I.; Lublinghoff, J.; Bircan, R.; Paschke, R. Genetics and phenomics of inherited and sporadic non-autoimmune hyperthyroidism. *Mol Cell Endocrinol* **2010**, *322*, 125-134, doi:10.1016/j.mce.2010.02.001.

44. Wasserman, J.D.; Sabbaghian, N.; Fahiminiya, S.; Chami, R.; Mete, O.; Acker, M.; Wu, M.K.; Shlien, A.; de Kock, L.; Foulkes, W.D. DICER1 Mutations Are Frequent in Adolescent-Onset Papillary Thyroid Carcinoma. *J Clin Endocrinol Metab* **2018**, *103*, 2009-2015, doi:10.1210/jc.2017-02698.

45. Cameselle-Teijeiro, J.M.; Mete, O.; Asa, S.L.; LiVolsi, V. Inherited Follicular Epithelial-Derived Thyroid Carcinomas: From Molecular Biology to Histological Correlates. *Endocr Pathol* **2021**, *32*, 77-101, doi:10.1007/s12022-020-09661-y.

46. Juhlin, C.C.; Stenman, A.; Zedenius, J. Macrofollicular variant follicular thyroid tumors are DICER1 mutated and exhibit distinct histological features. *Histopathology* **2021**, *79*, 661-666, doi:10.1111/his.14416.

47. Doerfler, W.R.; Nikitski, A.V.; Morariu, E.M.; Ohori, N.P.; Chiosea, S.I.; Landau, M.S.; Nikiforova, M.N.; Nikiforov, Y.E.; Yip, L.; Manroa, P. Molecular alterations in Hurthle cell nodules and preoperative cancer risk. *Endocr Relat Cancer* **2021**, *28*, 301-309, doi:10.1530/ERC-20-0435.

48. Hodak, S.; Tuttle, R.M.; Maytal, G.; Nikiforov, Y.E.; Randolph, G. Changing the Cancer Diagnosis: The Case of Follicular Variant of Papillary Thyroid Cancer-Primum Non Nocere and NIFTP. *Thyroid* **2016**, *26*, 869-871, doi:10.1089/thy.2016.0205.

49. Thompson, L.D. Ninety-four cases of encapsulated follicular variant of papillary thyroid carcinoma: A name change to Noninvasive Follicular Thyroid Neoplasm with Papillary-like Nuclear Features would help prevent overtreatment. *Mod Pathol* **2016**, *29*, 698-707, doi:10.1038/modpathol.2016.65.

50. Nikiforova, M.N.; Nikitski, A.V.; Panebianco, F.; Kaya, C.; Yip, L.; Williams, M.; Chiosea, S.I.; Seethala, R.R.; Roy, S.; Condello, V.; et al. GLIS Rearrangement is a Genomic Hallmark of Hyalinizing Trabecular Tumor of the Thyroid Gland. *Thyroid* **2019**, *29*, 161-173, doi:10.1089/thy.2018.0791.

51. Xu, B.; Wang, L.; Tuttle, R.M.; Ganly, I.; Ghossein, R. Prognostic impact of extent of vascular invasion in low-grade encapsulated follicular cell-derived thyroid carcinomas: a clinicopathologic study of 276 cases. *Hum Pathol* **2015**, *46*, 1789-1798, doi:10.1016/j.humpath.2015.08.015.

52. Hiltzik, D.; Carlson, D.L.; Tuttle, R.M.; Chuai, S.; Ishill, N.; Shaha, A.; Shah, J.P.; Singh, B.; Ghossein, R.A. Poorly differentiated thyroid carcinomas defined on the basis of mitosis and necrosis: a clinicopathologic study of 58 patients. *Cancer* **2006**, *106*, 1286-1295, doi:10.1002/cncr.21739.

53. Gnemmi, V.; Renaud, F.; Do Cao, C.; Salleron, J.; Lion, G.; Wemeau, J.L.; Copin, M.C.; Carnaille, B.; Leteurtre, E.; Pattou, F.; et al. Poorly differentiated thyroid carcinomas: application of the Turin proposal provides prognostic results similar to those from the assessment of high-grade features. *Histopathology* **2014**, *64*, 263-273, doi:10.1111/his.12246.

54. Volante, M.; Collini, P.; Nikiforov, Y.E.; Sakamoto, A.; Kakudo, K.; Katoh, R.; Lloyd, R.V.; LiVolsi, V.A.; Papotti, M.; Sobrinho-Simoes, M.; et al. Poorly differentiated thyroid carcinoma: the Turin proposal for the use of uniform diagnostic criteria and an algorithmic diagnostic approach. *Am J Surg Pathol* **2007**, *31*, 1256-1264, doi:10.1097/PAS.0b013e3180309e6a.

55. Wong, K.S.; Dong, F.; Telatar, M.; Lorch, J.H.; Alexander, E.K.; Marqusee, E.; Cho, N.L.; Nehs, M.A.; Doherty, G.M.; Afkhami, M.; et al. Papillary Thyroid Carcinoma with High-Grade Features Versus Poorly Differentiated Thyroid Carcinoma: An Analysis of Clinicopathologic and Molecular Features and Outcome. *Thyroid* **2021**, *31*, 933-940, doi:10.1089/thy.2020.0668.

56. Kakudo, K.; Bychkov, A.; Bai, Y.; Li, Y.; Liu, Z.; Jung, C.K. The new 4th edition World Health Organization classification for thyroid tumors, Asian perspectives. *Pathol Int* **2018**, *68*, 641-664, doi:10.1111/pin.12737.

57. Lam, K.Y.; Lo, C.Y.; Liu, M.C. Primary squamous cell carcinoma of the thyroid gland: an entity with aggressive clinical behaviour and distinctive cytokeratin expression profiles. *Histopathology* **2001**, *39*, 279-286, doi:10.1046/j.1365-2559.2001.01207.x.

58. Xu, B.; Fuchs, T.; Dogan, S.; Landa, I.; Katabi, N.; Fagin, J.A.; Tuttle, R.M.; Sherman, E.; Gill, A.J.; Ghossein, R. Dissecting Anaplastic Thyroid Carcinoma: A Comprehensive Clinical, Histologic, Immunophenotypic, and Molecular Study of 360 Cases. *Thyroid* **2020**, *30*, 1505-1517, doi:10.1089/thy.2020.0086.

59. Pelizzo, M.R.; Mazza, E.I.; Mian, C.; Merante Boschin, I. Medullary thyroid carcinoma. *Expert Rev Anticancer Ther* **2023**, *23*, 943-957, doi:10.1080/14737140.2023.2247566.

60. Alzumaili, B.; Xu, B.; Spanheimer, P.M.; Tuttle, R.M.; Sherman, E.; Katabi, N.; Dogan, S.; Ganly, I.; Untch, B.R.; Ghossein, R.A. Grading of medullary thyroid carcinoma on the basis of tumor necrosis and high mitotic rate is an independent predictor of poor outcome. *Mod Pathol* **2020**, *33*, 1690-1701, doi:10.1038/s41379-020-0532-1.

61. Sahin, M.; Oguz, A.; Tuzun, D.; Akkus, G.; Torun, G.I.; Bahar, A.Y.; Sahin, H.; Gul, K. Effectiveness of TI-RADS and ATA classifications for predicting malignancy of thyroid nodules. *Adv Clin Exp Med* **2021**, *30*, 1133-1139, doi:10.17219/acem/139591.

62. Haugen, B.R.; Alexander, E.K.; Bible, K.C.; Doherty, G.M.; Mandel, S.J.; Nikiforov, Y.E.; Pacini, F.; Randolph, G.W.; Sawka, A.M.; Schlumberger, M.; et al. 2015 American Thyroid Association Management Guidelines for Adult Patients with Thyroid Nodules and Differentiated Thyroid Cancer: The American Thyroid Association Guidelines Task Force on Thyroid Nodules and Differentiated Thyroid Cancer. *Thyroid* **2016**, *26*, 1-133, doi:10.1089/thy.2015.0020.

63. Cibas, E.S.; Ali, S.Z. The 2017 Bethesda System for Reporting Thyroid Cytopathology. *Thyroid* **2017**, *27*, 1341-1346, doi:10.1089/thy.2017.0500.

64. Maletta, F.; Massa, F.; Torregrossa, L.; Duregon, E.; Casadei, G.P.; Basolo, F.; Tallini, G.; Volante, M.; Nikiforov, Y.E.; Papotti, M. Cytological features of "noninvasive follicular thyroid neoplasm with papillary-like nuclear features" and their correlation with tumor histology. *Hum Pathol* **2016**, *54*, 134-142, doi:10.1016/j.humpath.2016.03.014.

65. Pizzato, M.; Li, M.; Vignat, J.; Laversanne, M.; Singh, D.; La Vecchia, C.; Vaccarella, S. The epidemiological landscape of thyroid cancer worldwide: GLOBOCAN estimates for incidence and mortality rates in 2020. *Lancet Diabetes Endocrinol* **2022**, *10*, 264-272, doi:10.1016/S2213-8587(22)00035-3.

66. Lamartina, L.; Leboulleux, S.; Borget, I.; Schlumberger, M. Global thyroid estimates in 2020. *Lancet Diabetes Endocrinol* **2022**, *10*, 235-236, doi:10.1016/S2213-8587(22)00048-1.

67. Li, M.; Dal Maso, L.; Vaccarella, S. Global trends in thyroid cancer incidence and the impact of overdiagnosis. *Lancet Diabetes Endocrinol* **2020**, *8*, 468-470, doi:10.1016/S2213-8587(20)30115-7.

68. Vaccarella, S.; Franceschi, S.; Bray, F.; Wild, C.P.; Plummer, M.; Dal Maso, L. Worldwide Thyroid-Cancer Epidemic? The Increasing Impact of Overdiagnosis. *N Engl J Med* **2016**, *375*, 614-617, doi:10.1056/NEJMp1604412.

69. Surveillance Research Program, N. SEER* Explorer: an interactive website for SEER cancer statistics. **2021**.

70. Saad, M.F.; Ordonez, N.G.; Rashid, R.K.; Guido, J.J.; Hill, C.S., Jr.; Hickey, R.C.; Samaan, N.A. Medullary carcinoma of the thyroid. A study of the clinical features and prognostic factors in 161 patients. *Medicine (Baltimore)* **1984**, *63*, 319-342.

71. Are, C.; Shaha, A.R. Anaplastic thyroid carcinoma: biology, pathogenesis, prognostic factors, and treatment approaches. *Ann Surg Oncol* **2006**, *13*, 453-464, doi:10.1245/ASO.2006.05.042.

72. Untch, B.R.; Olson, J.A., Jr. Anaplastic thyroid carcinoma, thyroid lymphoma, and metastasis to thyroid. *Surg Oncol Clin N Am* **2006**, *15*, 661-679, x, doi:10.1016/j.soc.2006.05.006.

73. Brierley, J.; Tsang, R.; Simpson, W.J.; Gospodarowicz, M.; Sutcliffe, S.; Panzarella, T. Medullary thyroid cancer: analyses of survival and prognostic factors and the role of radiation therapy in local control. *Thyroid* **1996**, *6*, 305-310, doi:10.1089/thy.1996.6.305.

74. Lutz, S.; Berk, L.; Chang, E.; Chow, E.; Hahn, C.; Hoskin, P.; Howell, D.; Konski, A.; Kachnic, L.; Lo, S.; et al. Palliative radiotherapy for bone metastases: an ASTRO evidence-

based guideline. *Int J Radiat Oncol Biol Phys* **2011**, *79*, 965-976, doi:10.1016/j.ijrobp.2010.11.026.

75. Subbiah, V.; Kreitman, R.J.; Wainberg, Z.A.; Cho, J.Y.; Schellens, J.H.M.; Soria, J.C.; Wen, P.Y.; Zielinski, C.; Cabanillas, M.E.; Urbanowitz, G.; et al. Dabrafenib and Trametinib Treatment in Patients With Locally Advanced or Metastatic BRAF V600-Mutant Anaplastic Thyroid Cancer. *J Clin Oncol* **2018**, *36*, 7-13, doi:10.1200/JCO.2017.73.6785.

76. Drilon, A.; Laetsch, T.W.; Kummar, S.; DuBois, S.G.; Lassen, U.N.; Demetri, G.D.; Nathenson, M.; Doebele, R.C.; Farago, A.F.; Pappo, A.S.; et al. Efficacy of Larotrectinib in TRK Fusion-Positive Cancers in Adults and Children. *N Engl J Med* **2018**, *378*, 731-739, doi:10.1056/NEJMoa1714448.

77. Doebele, R.C.; Drilon, A.; Paz-Ares, L.; Siena, S.; Shaw, A.T.; Farago, A.F.; Blakely, C.M.; Seto, T.; Cho, B.C.; Tosi, D.; et al. Entrectinib in patients with advanced or metastatic NTRK fusion-positive solid tumours: integrated analysis of three phase 1-2 trials. *Lancet Oncol* **2020**, *21*, 271-282, doi:10.1016/S1470-2045(19)30691-6.

78. Wirth, L.J.; Sherman, E.; Robinson, B.; Solomon, B.; Kang, H.; Lorch, J.; Worden, F.; Brose, M.; Patel, J.; Leboulleux, S.; et al. Efficacy of Selpercatinib in RET-Altered Thyroid Cancers. *N Engl J Med* **2020**, *383*, 825-835, doi:10.1056/NEJMoa2005651.

79. Subbiah, V.; Hu, M.I.; Wirth, L.J.; Schuler, M.; Mansfield, A.S.; Curigliano, G.; Brose, M.S.; Zhu, V.W.; Leboulleux, S.; Bowles, D.W.; et al. Pralsetinib for patients with advanced or metastatic RET-altered thyroid cancer (ARROW): a multi-cohort, open-label, registrational, phase 1/2 study. *Lancet Diabetes Endocrinol* **2021**, *9*, 491-501, doi:10.1016/S2213-8587(21)00120-0.

80. Marabelle, A.; Fakih, M.; Lopez, J.; Shah, M.; Shapira-Frommer, R.; Nakagawa, K.; Chung, H.C.; Kindler, H.L.; Lopez-Martin, J.A.; Miller, W.H., Jr.; et al. Association of tumour mutational burden with outcomes in patients with advanced solid tumours treated with pembrolizumab: prospective biomarker analysis of the multicohort, open-label, phase 2

KEYNOTE-158 study. *Lancet Oncol* **2020**, *21*, 1353-1365, doi:10.1016/S1470-2045(20)30445-9.

81. Bible, K.C.; Kebebew, E.; Brierley, J.; Brito, J.P.; Cabanillas, M.E.; Clark, T.J., Jr.; Di Cristofano, A.; Foote, R.; Giordano, T.; Kasperbauer, J.; et al. 2021 American Thyroid Association Guidelines for Management of Patients with Anaplastic Thyroid Cancer. *Thyroid* **2021**, *31*, 337-386, doi:10.1089/thy.2020.0944.

82. Shimaoka, K.; Schoenfeld, D.A.; DeWys, W.D.; Creech, R.H.; DeConti, R. A randomized trial of doxorubicin versus doxorubicin plus cisplatin in patients with advanced thyroid carcinoma. *Cancer* **1985**, *56*, 2155-2160, doi:10.1002/1097-0142(19851101)56:9<2155::aid-cncr2820560903>3.0.co;2-e.

83. Higashiyama, T.; Ito, Y.; Hirokawa, M.; Fukushima, M.; Uruno, T.; Miya, A.; Matsuzuka, F.; Miyauchi, A. Induction chemotherapy with weekly paclitaxel administration for anaplastic thyroid carcinoma. *Thyroid* **2010**, *20*, 7-14, doi:10.1089/thy.2009.0115.

84. Sosa, J.A.; Balkissoon, J.; Lu, S.P.; Langecker, P.; Elisei, R.; Jarzab, B.; Bal, C.S.; Marur, S.; Gramza, A.; Ondrey, F. Thyroidectomy followed by fosbretabulin (CA4P) combination regimen appears to suggest improvement in patient survival in anaplastic thyroid cancer. *Surgery* **2012**, *152*, 1078-1087, doi:10.1016/j.surg.2012.08.036.

85. Bible, K.C.; Suman, V.J.; Menefee, M.E.; Smallridge, R.C.; Molina, J.R.; Maples, W.J.; Karlin, N.J.; Traynor, A.M.; Kumar, P.; Goh, B.C.; et al. A multiinstitutional phase 2 trial of pazopanib monotherapy in advanced anaplastic thyroid cancer. *J Clin Endocrinol Metab* **2012**, *97*, 3179-3184, doi:10.1210/jc.2012-1520.

86. Ha, H.T.; Lee, J.S.; Urba, S.; Koenig, R.J.; Sisson, J.; Giordano, T.; Worden, F.P. A phase II study of imatinib in patients with advanced anaplastic thyroid cancer. *Thyroid* **2010**, *20*, 975-980, doi:10.1089/thy.2010.0057.

87. Ravaud, A.; de la Fouchardiere, C.; Caron, P.; Doussau, A.; Do Cao, C.; Asselineau, J.; Rodien, P.; Pouessel, D.; Nicolli-Sire, P.; Klein, M.; et al. A multicenter phase II study of

sunitinib in patients with locally advanced or metastatic differentiated, anaplastic or medullary thyroid carcinomas: mature data from the THYSU study. *Eur J Cancer* **2017**, *76*, 110-117, doi:10.1016/j.ejca.2017.01.029.

88. Wu, X.; Zhou, Q.H.; Xu, K. Are isothiocyanates potential anti-cancer drugs? *Acta Pharmacol Sin* **2009**, *30*, 501-512, doi:10.1038/aps.2009.50.

89. Lam, T.K.; Gallicchio, L.; Lindsley, K.; Shiels, M.; Hammond, E.; Tao, X.G.; Chen, L.; Robinson, K.A.; Caulfield, L.E.; Herman, J.G.; et al. Cruciferous vegetable consumption and lung cancer risk: a systematic review. *Cancer Epidemiol Biomarkers Prev* **2009**, *18*, 184-195, doi:10.1158/1055-9965.EPI-08-0710.

90. Wolf, M.A.; Claudio, P.P. Benzyl isothiocyanate inhibits HNSCC cell migration and invasion, and sensitizes HNSCC cells to cisplatin. *Nutr Cancer* **2014**, *66*, 285-294, doi:10.1080/01635581.2014.868912.

91. Murata, M.; Yamashita, N.; Inoue, S.; Kawanishi, S. Mechanism of oxidative DNA damage induced by carcinogenic allyl isothiocyanate. *Free Radic Biol Med* **2000**, *28*, 797-805, doi:10.1016/s0891-5849(00)00168-4.

92. De Gianni, E.; Fimognari, C. Anticancer Mechanism of Sulfur-Containing Compounds. *Enzymes* **2015**, *37*, 167-192, doi:10.1016/bs.enz.2015.05.003.

93. Nakamura, Y.; Yoshimoto, M.; Murata, Y.; Shimoishi, Y.; Asai, Y.; Park, E.Y.; Sato, K.; Nakamura, Y. Papaya seed represents a rich source of biologically active isothiocyanate. *J Agric Food Chem* **2007**, *55*, 4407-4413, doi:10.1021/jf070159w.

94. Dufour, V.; Alazzam, B.; Ermel, G.; Thepaut, M.; Rossero, A.; Tresse, O.; Baysse, C. Antimicrobial activities of isothiocyanates against Campylobacter jejuni isolates. *Front Cell Infect Microbiol* **2012**, *2*, 53, doi:10.3389/fcimb.2012.00053.

95. Dinh, T.N.; Parat, M.O.; Ong, Y.S.; Khaw, K.Y. Anticancer activities of dietary benzyl isothiocyanate: A comprehensive review. *Pharmacol Res* **2021**, *169*, 105666, doi:10.1016/j.phrs.2021.105666.

96. Tang, N.Y.; Chueh, F.S.; Yu, C.C.; Liao, C.L.; Lin, J.J.; Hsia, T.C.; Wu, K.C.; Liu, H.C.; Lu, K.W.; Chung, J.G. Benzyl isothiocyanate alters the gene expression with cell cycle regulation and cell death in human brain glioblastoma GBM 8401 cells. *Oncol Rep* **2016**, *35*, 2089-2096, doi:10.3892/or.2016.4577.

97. Kim, S.H.; Singh, S.V. p53-Independent apoptosis by benzyl isothiocyanate in human breast cancer cells is mediated by suppression of XIAP expression. *Cancer Prev Res (Phila)* **2010**, *3*, 718-726, doi:10.1158/1940-6207.CAPR-10-0048.

98. Zhou, T.; Li, G.; Cao, B.; Liu, L.; Cheng, Q.; Kong, H.; Shan, C.; Huang, X.; Chen, J.; Gao, N. Downregulation of Mcl-1 through inhibition of translation contributes to benzyl isothiocyanate-induced cell cycle arrest and apoptosis in human leukemia cells. *Cell Death Dis* **2013**, *4*, e515, doi:10.1038/cddis.2013.41.

99. Zhu, M.; Li, W.; Guo, J.; Lu, Y.; Dong, X.; Lin, B.; Chen, Y.; Zhang, X.; Li, M. Alpha fetoprotein antagonises benzyl isothiocyanate inhibition of the malignant behaviors of hepatocellular carcinoma cells. *Oncotarget* **2016**, *7*, 75749-75762, doi:10.18632/oncotarget.12407.

100. Tsou, M.F.; Peng, C.T.; Shih, M.C.; Yang, J.S.; Lu, C.C.; Chiang, J.H.; Wu, C.L.; Lin, J.P.; Lo, C.; Fan, M.J.; et al. Benzyl isothiocyanate inhibits murine WEHI-3 leukemia cells in vitro and promotes phagocytosis in BALB/c mice in vivo. *Leuk Res* **2009**, *33*, 1505-1511, doi:10.1016/j.leukres.2009.01.030.

101. Huang, S.H.; Wu, L.W.; Huang, A.C.; Yu, C.C.; Lien, J.C.; Huang, Y.P.; Yang, J.S.; Yang, J.H.; Hsiao, Y.P.; Wood, W.G.; et al. Benzyl isothiocyanate (BITC) induces G2/M phase arrest and apoptosis in human melanoma A375.S2 cells through reactive oxygen species (ROS) and both mitochondria-dependent and death receptor-mediated multiple signaling pathways. *J Agric Food Chem* **2012**, *60*, 665-675, doi:10.1021/jf204193v.

102. Kasiappan, R.; Jutooru, I.; Karki, K.; Hedrick, E.; Safe, S. Benzyl Isothiocyanate (BITC) Induces Reactive Oxygen Species-dependent Repression of STAT3 Protein by Down-

regulation of Specificity Proteins in Pancreatic Cancer. *J Biol Chem* **2016**, *291*, 27122-27133, doi:10.1074/jbc.M116.746339.

103. Han, K.W.W.; Po, W.W.; Sohn, U.D.; Kim, H.J. Benzyl Isothiocyanate Induces Apoptosis via Reactive Oxygen Species-Initiated Mitochondrial Dysfunction and DR4 and DR5 Death Receptor Activation in Gastric Adenocarcinoma Cells. *Biomolecules* **2019**, *9*, doi:10.3390/biom9120839.

104. Sehrawat, A.; Croix, C.S.; Baty, C.J.; Watkins, S.; Tailor, D.; Singh, R.P.; Singh, S.V. Inhibition of mitochondrial fusion is an early and critical event in breast cancer cell apoptosis by dietary chemopreventative benzyl isothiocyanate. *Mitochondrion* **2016**, *30*, 67-77, doi:10.1016/j.mito.2016.06.006.

105. Ma, Y.S.; Lin, J.J.; Lin, C.C.; Lien, J.C.; Peng, S.F.; Fan, M.J.; Hsu, F.T.; Chung, J.G. Benzyl isothiocyanate inhibits human brain glioblastoma multiforme GBM 8401 cell xenograft tumor in nude mice in vivo. *Environ Toxicol* **2018**, *33*, 1097-1104, doi:10.1002/tox.22581.

106. Antony, M.L.; Kim, S.H.; Singh, S.V. Critical role of p53 upregulated modulator of apoptosis in benzyl isothiocyanate-induced apoptotic cell death. *PLoS One* **2012**, *7*, e32267, doi:10.1371/journal.pone.0032267.

107. Stasilojc, G.; Nagel, A.; Koszalka, P.; Bigda, J.J. Defective apoptosis of U937 cells induced by benzyl isothiocyanate (BITC). *Acta Biochim Pol* **2019**, *66*, 401-407, doi:10.18388/abp.2019_2769.

108. Ma, L.; Chen, Y.; Han, R.; Wang, S. Benzyl isothiocyanate inhibits invasion and induces apoptosis via reducing S100A4 expression and increases PUMA expression in oral squamous cell carcinoma cells. *Braz J Med Biol Res* **2019**, *52*, e8409, doi:10.1590/1414-431X20198409.

109. Shang, H.S.; Shih, Y.L.; Lu, T.J.; Lee, C.H.; Hsueh, S.C.; Chou, Y.C.; Lu, H.F.; Liao, N.C.; Chung, J.G. Benzyl isothiocyanate (BITC) induces apoptosis of GBM 8401 human

brain glioblastoma multiforms cells via activation of caspase-8/Bid and the reactive oxygen species-dependent mitochondrial pathway. *Environ Toxicol* **2016**, *31*, 1751-1760, doi:10.1002/tox.22177.

110. Zhu, Y.; Liu, A.; Zhang, X.; Qi, L.; Zhang, L.; Xue, J.; Liu, Y.; Yang, P. The effect of benzyl isothiocyanate and its computer-aided design derivants targeting alkylglycerone phosphate synthase on the inhibition of human glioma U87MG cell line. *Tumour Biol* **2015**, *36*, 3499-3509, doi:10.1007/s13277-014-2986-6.

111. Wicker, C.A.; Sahu, R.P.; Kulkarni-Datar, K.; Srivastava, S.K.; Brown, T.L. BITC Sensitizes Pancreatic Adenocarcinomas to TRAIL-induced Apoptosis. *Cancer Growth Metastasis* **2010**, *2009*, 45-55.

112. Besson, A.; Dowdy, S.F.; Roberts, J.M. CDK inhibitors: cell cycle regulators and beyond. *Dev Cell* **2008**, *14*, 159-169, doi:10.1016/j.devcel.2008.01.013.

113. Otto, T.; Sicinski, P. Cell cycle proteins as promising targets in cancer therapy. *Nat Rev Cancer* **2017**, *17*, 93-115, doi:10.1038/nrc.2016.138.

114. Mi, L.; Gan, N.; Chung, F.L. Isothiocyanates inhibit proteasome activity and proliferation of multiple myeloma cells. *Carcinogenesis* **2011**, *32*, 216-223, doi:10.1093/carcin/bgq242.

115. Zhang, R.; Loganathan, S.; Humphreys, I.; Srivastava, S.K. Benzyl isothiocyanate-induced DNA damage causes G2/M cell cycle arrest and apoptosis in human pancreatic cancer cells. *J Nutr* **2006**, *136*, 2728-2734, doi:10.1093/jn/136.11.2728.

116. Koch, S.; Claesson-Welsh, L. Signal transduction by vascular endothelial growth factor receptors. *Cold Spring Harb Perspect Med* **2012**, *2*, a006502, doi:10.1101/cshperspect.a006502.

117. Warin, R.; Xiao, D.; Arlotti, J.A.; Bommareddy, A.; Singh, S.V. Inhibition of human breast cancer xenograft growth by cruciferous vegetable constituent benzyl isothiocyanate. *Mol Carcinog* **2010**, *49*, 500-507, doi:10.1002/mc.20600.

118. Lamouille, S.; Xu, J.; Derynck, R. Molecular mechanisms of epithelial-mesenchymal transition. *Nat Rev Mol Cell Biol* **2014**, *15*, 178-196, doi:10.1038/nrm3758.

119. Loh, C.Y.; Chai, J.Y.; Tang, T.F.; Wong, W.F.; Sethi, G.; Shanmugam, M.K.; Chong, P.P.; Looi, C.Y. The E-Cadherin and N-Cadherin Switch in Epithelial-to-Mesenchymal Transition: Signaling, Therapeutic Implications, and Challenges. *Cells* **2019**, *8*, doi:10.3390/cells8101118.

120. Sehrawat, A.; Kim, S.H.; Vogt, A.; Singh, S.V. Suppression of FOXQ1 in benzyl isothiocyanate-mediated inhibition of epithelial-mesenchymal transition in human breast cancer cells. *Carcinogenesis* **2013**, *34*, 864-873, doi:10.1093/carcin/bgs397.

121. Kim, S.H.; Nagalingam, A.; Saxena, N.K.; Singh, S.V.; Sharma, D. Benzyl isothiocyanate inhibits oncogenic actions of leptin in human breast cancer cells by suppressing activation of signal transducer and activator of transcription 3. *Carcinogenesis* **2011**, *32*, 359-367, doi:10.1093/carcin/bgq267.

122. Pore, S.K.; Hahm, E.R.; Latoche, J.D.; Anderson, C.J.; Shuai, Y.; Singh, S.V. Prevention of breast cancer-induced osteolytic bone resorption by benzyl isothiocyanate. *Carcinogenesis* **2018**, *39*, 134-145, doi:10.1093/carcin/bgx114.

123. Lu, P.; Takai, K.; Weaver, V.M.; Werb, Z. Extracellular matrix degradation and remodeling in development and disease. *Cold Spring Harb Perspect Biol* **2011**, *3*, doi:10.1101/cshperspect.a005058.

124. Lee, C.S.; Cho, H.J.; Jeong, Y.J.; Shin, J.M.; Park, K.K.; Park, Y.Y.; Bae, Y.S.; Chung, I.K.; Kim, M.; Kim, C.H.; et al. Isothiocyanates inhibit the invasion and migration of C6 glioma cells by blocking FAK/JNK-mediated MMP-9 expression. *Oncol Rep* **2015**, *34*, 2901-2908, doi:10.3892/or.2015.4292.

125. Jeong, Y.J.; Cho, H.J.; Chung, F.L.; Wang, X.; Hoe, H.S.; Park, K.K.; Kim, C.H.; Chang, H.W.; Lee, S.R.; Chang, Y.C. Isothiocyanates suppress the invasion and metastasis of

tumors by targeting FAK/MMP-9 activity. *Oncotarget* **2017**, *8*, 63949-63962, doi:10.18632/oncotarget.19213.

126. Guo, Y.J.; Pan, W.W.; Liu, S.B.; Shen, Z.F.; Xu, Y.; Hu, L.L. ERK/MAPK signalling pathway and tumorigenesis. *Exp Ther Med* **2020**, *19*, 1997-2007, doi:10.3892/etm.2020.8454.

127. Tang, Q.; Wu, J.; Zheng, F.; Hann, S.S.; Chen, Y. Emodin Increases Expression of Insulin-Like Growth Factor Binding Protein 1 through Activation of MEK/ERK/AMPKalpha and Interaction of PPARgamma and Sp1 in Lung Cancer. *Cell Physiol Biochem* **2017**, *41*, 339-357, doi:10.1159/000456281.

128. Lui, V.W.; Wentzel, A.L.; Xiao, D.; Lew, K.L.; Singh, S.V.; Grandis, J.R. Requirement of a carbon spacer in benzyl isothiocyanate-mediated cytotoxicity and MAPK activation in head and neck squamous cell carcinoma. *Carcinogenesis* **2003**, *24*, 1705-1712, doi:10.1093/carcin/bgg127.

129. Sahu, R.P.; Zhang, R.; Batra, S.; Shi, Y.; Srivastava, S.K. Benzyl isothiocyanate-mediated generation of reactive oxygen species causes cell cycle arrest and induces apoptosis via activation of MAPK in human pancreatic cancer cells. *Carcinogenesis* **2009**, *30*, 1744-1753, doi:10.1093/carcin/bgp157.

130. Xiao, D.; Powolny, A.A.; Singh, S.V. Benzyl isothiocyanate targets mitochondrial respiratory chain to trigger reactive oxygen species-dependent apoptosis in human breast cancer cells. *J Biol Chem* **2008**, *283*, 30151-30163, doi:10.1074/jbc.M802529200.

131. Ho, C.C.; Lai, K.C.; Hsu, S.C.; Kuo, C.L.; Ma, C.Y.; Lin, M.L.; Yang, J.S.; Chung, J.G. Benzyl isothiocyanate (BITC) inhibits migration and invasion of human gastric cancer AGS cells via suppressing ERK signal pathways. *Hum Exp Toxicol* **2011**, *30*, 296-306, doi:10.1177/0960327110371991.

132. Hwang, E.S.; Lee, H.J. Benzyl isothiocyanate inhibits metalloproteinase-2/-9 expression by suppressing the mitogen-activated protein kinase in SK-Hep1 human hepatoma cells. *Food Chem Toxicol* **2008**, *46*, 2358-2364, doi:10.1016/j.fct.2008.03.016.

133. Xiao, D.; Bommareddy, A.; Kim, S.H.; Sehrawat, A.; Hahm, E.R.; Singh, S.V. Benzyl isothiocyanate causes FoxO1-mediated autophagic death in human breast cancer cells. *PLoS One* **2012**, *7*, e32597, doi:10.1371/journal.pone.0032597.

134. Boreddy, S.R.; Pramanik, K.C.; Srivastava, S.K. Pancreatic tumor suppression by benzyl isothiocyanate is associated with inhibition of PI3K/AKT/FOXO pathway. *Clin Cancer Res* **2011**, *17*, 1784-1795, doi:10.1158/1078-0432.CCR-10-1891.

135. Carnero, A.; Paramio, J.M. The PTEN/PI3K/AKT Pathway in vivo, Cancer Mouse Models. *Front Oncol* **2014**, *4*, 252, doi:10.3389/fonc.2014.00252.

136. Panigrahi, A.R.; Pinder, S.E.; Chan, S.Y.; Paish, E.C.; Robertson, J.F.; Ellis, I.O. The role of PTEN and its signalling pathways, including AKT, in breast cancer; an assessment of relationships with other prognostic factors and with outcome. *J Pathol* **2004**, *204*, 93-100, doi:10.1002/path.1611.

137. Yang, Q.; Miyagawa, M.; Liu, X.; Zhu, B.; Munemasa, S.; Nakamura, T.; Murata, Y.; Nakamura, Y. Methyl-beta-cyclodextrin potentiates the BITC-induced anti-cancer effect through modulation of the Akt phosphorylation in human colorectal cancer cells. *Biosci Biotechnol Biochem* **2018**, *82*, 2158-2167, doi:10.1080/09168451.2018.1514249.

138. Liu, X.; Takano, C.; Shimizu, T.; Yokobe, S.; Abe-Kanoh, N.; Zhu, B.; Nakamura, T.; Munemasa, S.; Murata, Y.; Nakamura, Y. Inhibition of phosphatidylinositide 3-kinase ameliorates antiproliferation by benzyl isothiocyanate in human colon cancer cells. *Biochem Biophys Res Commun* **2017**, *491*, 209-216, doi:10.1016/j.bbrc.2017.07.078.

139. Munoz-Espin, D.; Serrano, M. Cellular senescence: from physiology to pathology. *Nat Rev Mol Cell Biol* **2014**, *15*, 482-496, doi:10.1038/nrm3823.

140. He, S.; Sharpless, N.E. Senescence in Health and Disease. *Cell* **2017**, *169*, 1000-1011, doi:10.1016/j.cell.2017.05.015.

141. Wei, W.; Ji, S. Cellular senescence: Molecular mechanisms and pathogenicity. *J Cell Physiol* **2018**, *233*, 9121-9135, doi:10.1002/jcp.26956.

142. Hinds, P.; Pietruska, J. Senescence and tumor suppression. *F1000Res* **2017**, *6*, 2121, doi:10.12688/f1000research.11671.1.

143. Rodier, F.; Coppe, J.P.; Patil, C.K.; Hoeijmakers, W.A.; Munoz, D.P.; Raza, S.R.; Freund, A.; Campeau, E.; Davalos, A.R.; Campisi, J. Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion. *Nat Cell Biol* **2009**, *11*, 973-979, doi:10.1038/ncb1909.

144. Coppe, J.P.; Patil, C.K.; Rodier, F.; Sun, Y.; Munoz, D.P.; Goldstein, J.; Nelson, P.S.; Desprez, P.Y.; Campisi, J. Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. *PLoS Biol* **2008**, *6*, 2853-2868, doi:10.1371/journal.pbio.0060301.

145. Malavolta, M.; Bracci, M.; Santarelli, L.; Sayeed, M.A.; Pierpaoli, E.; Giacconi, R.; Costarelli, L.; Piacenza, F.; Basso, A.; Cardelli, M.; et al. Inducers of Senescence, Toxic Compounds, and Senolytics: The Multiple Faces of Nrf2-Activating Phytochemicals in Cancer Adjuvant Therapy. *Mediators Inflamm* **2018**, *2018*, 4159013, doi:10.1155/2018/4159013.

146. Roy, N.; Elangovan, I.; Kopanja, D.; Bagchi, S.; Raychaudhuri, P. Tumor regression by phenethyl isothiocyanate involves DDB2. *Cancer Biol Ther* **2013**, *14*, 108-116, doi:10.4161/cbt.22631.

147. Aggarwal, M.; Saxena, R.; Sinclair, E.; Fu, Y.; Jacobs, A.; Dyba, M.; Wang, X.; Cruz, I.; Berry, D.; Kallakury, B.; et al. Reactivation of mutant p53 by a dietary-related compound phenethyl isothiocyanate inhibits tumor growth. *Cell Death Differ* **2016**, *23*, 1615-1627, doi:10.1038/cdd.2016.48.

148. Sarkars, R.; Mukherjee, S.; Roy, M. Targeting heat shock proteins by phenethyl isothiocyanate results in cell-cycle arrest and apoptosis of human breast cancer cells. *Nutr Cancer* **2013**, *65*, 480-493, doi:10.1080/01635581.2013.767366.

149. Yeh, Y.T.; Yeh, H.; Su, S.H.; Lin, J.S.; Lee, K.J.; Shyu, H.W.; Chen, Z.F.; Huang, S.Y.; Su, S.J. Phenethyl isothiocyanate induces DNA damage-associated G2/M arrest and subsequent apoptosis in oral cancer cells with varying p53 mutations. *Free Radic Biol Med* **2014**, *74*, 1-13, doi:10.1016/j.freeradbiomed.2014.06.008.

150. Zheng, K.; Ma, J.; Wang, Y.; He, Z.; Deng, K. Sulforaphane Inhibits Autophagy and Induces Exosome-Mediated Paracrine Senescence via Regulating mTOR/TFE3. *Mol Nutr Food Res* **2020**, *64*, e1901231, doi:10.1002/mnfr.201901231.

151. Calcinotto, A.; Kohli, J.; Zagato, E.; Pellegrini, L.; Demaria, M.; Alimonti, A. Cellular Senescence: Aging, Cancer, and Injury. *Physiol Rev* **2019**, *99*, 1047-1078, doi:10.1152/physrev.00020.2018.

152. Hill, M.; Tran, N. miRNA interplay: mechanisms and consequences in cancer. *Dis Model Mech* **2021**, *14*, doi:10.1242/dmm.047662.

153. He, B.; Zhao, Z.; Cai, Q.; Zhang, Y.; Zhang, P.; Shi, S.; Xie, H.; Peng, X.; Yin, W.; Tao, Y.; et al. miRNA-based biomarkers, therapies, and resistance in Cancer. *Int J Biol Sci* **2020**, *16*, 2628-2647, doi:10.7150/ijbs.47203.

154. Lee, Y.S.; Dutta, A. MicroRNAs in cancer. *Annu Rev Pathol* **2009**, *4*, 199-227, doi:10.1146/annurev.pathol.4.110807.092222.

155. Leonardi, G.C.; Candido, S.; Carbone, M.; Colaianni, V.; Garozzo, S.F.; Cina, D.; Libra, M. microRNAs and thyroid cancer: biological and clinical significance (Review). *Int J Mol Med* **2012**, *30*, 991-999, doi:10.3892/ijmm.2012.1089.

156. Huang, H.G.; Luo, X.; Wu, S.; Jian, B. MiR-99a Inhibits Cell Proliferation and Tumorigenesis through Targeting mTOR in Human Anaplastic Thyroid Cancer. *Asian Pac J Cancer Prev* **2015**, *16*, 4937-4944, doi:10.7314/apjcp.2015.16.12.4937.

157. Gao, Y.; Pan, Y.; Wang, T.; Yao, Y.; Yuan, W.; Zhu, X.; Wang, K. MicroRNA-99a-3p/GRP94 axis affects metastatic progression of human papillary thyroid carcinoma by

regulating ITGA2 expression and localization. *Acta Biochim Biophys Sin (Shanghai)* **2021**, *53*, 1650-1661, doi:10.1093/abbs/gmab147.

158. Tsai, T.F.; Chen, P.C.; Lin, Y.C.; Chou, K.Y.; Chen, H.E.; Ho, C.Y.; Lin, J.F.; Hwang, T.I. Benzyl isothiocyanate promotes miR-99a expression through ERK/AP-1-dependent pathway in bladder cancer cells. *Environ Toxicol* **2020**, *35*, 47-54, doi:10.1002/tox.22841.

159. Scuderi, S.A.; Casili, G.; Filippone, A.; Lanza, M.; Basilotta, R.; Giuffrida, R.; Munao, S.; Colarossi, L.; Capra, A.P.; Esposito, E.; et al. Beneficial effect of KYP-2047, a propyl-oligopeptidase inhibitor, on oral squamous cell carcinoma. *Oncotarget* **2021**, *12*, 2459-2473, doi:10.18632/oncotarget.28147.

160. Scuderi, S.A.; Casili, G.; Basilotta, R.; Lanza, M.; Filippone, A.; Raciti, G.; Puliafito, I.; Colarossi, L.; Esposito, E.; Paterniti, I. NLRP3 Inflammasome Inhibitor BAY-117082 Reduces Oral Squamous Cell Carcinoma Progression. *Int J Mol Sci* **2021**, *22*, doi:10.3390/ijms222011108.

161. Liang, C.C.; Park, A.Y.; Guan, J.L. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nat Protoc* **2007**, *2*, 329-333, doi:10.1038/nprot.2007.30.

162. Cheng, N.; Diao, H.; Lin, Z.; Gao, J.; Zhao, Y.; Zhang, W.; Wang, Q.; Lin, J.; Zhang, D.; Jin, Y.; et al. Benzyl Isothiocyanate Induces Apoptosis and Inhibits Tumor Growth in Canine Mammary Carcinoma via Downregulation of the Cyclin B1/Cdk1 Pathway. *Front Vet Sci* **2020**, *7*, 580530, doi:10.3389/fvets.2020.580530.

163. Troiani, M.; Colucci, M.; D'Ambrosio, M.; Guccini, I.; Pasquini, E.; Varesi, A.; Valdata, A.; Mosole, S.; Revandkar, A.; Attanasio, G.; et al. Single-cell transcriptomics identifies Mcl-1 as a target for senolytic therapy in cancer. *Nat Commun* **2022**, *13*, 2177, doi:10.1038/s41467-022-29824-1.

164. Guccini, I.; Revandkar, A.; D'Ambrosio, M.; Colucci, M.; Pasquini, E.; Mosole, S.; Troiani, M.; Brina, D.; Sheibani-Tezerji, R.; Elia, A.R.; et al. Senescence Reprogramming by

TIMP1 Deficiency Promotes Prostate Cancer Metastasis. *Cancer Cell* **2021**, *39*, 68-82 e69, doi:10.1016/j.ccell.2020.10.012.

165. Sewell, W.; Reeb, A.; Lin, R.Y. An orthotopic mouse model of anaplastic thyroid carcinoma. *J Vis Exp* **2013**, doi:10.3791/50097.

166. Fanfone, D.; Stanicki, D.; Nonclercq, D.; Port, M.; Vander Elst, L.; Laurent, S.; Muller, R.N.; Saussez, S.; Burtea, C. Molecular Imaging of Galectin-1 Expression as a Biomarker of Papillary Thyroid Cancer by Using Peptide-Functionalized Imaging Probes. *Biology (Basel)* **2020**, *9*, doi:10.3390/biology9030053.

167. Casili, G.; Caffo, M.; Campolo, M.; Barresi, V.; Caruso, G.; Cardali, S.M.; Lanza, M.; Mallamace, R.; Filippone, A.; Conti, A.; et al. TLR-4/Wnt modulation as new therapeutic strategy in the treatment of glioblastomas. *Oncotarget* **2018**, *9*, 37564-37580, doi:10.18632/oncotarget.26500.

168. Basilotta, R.; Lanza, M.; Filippone, A.; Casili, G.; Mannino, D.; De Gaetano, F.; Chisari, G.; Colarossi, L.; Motta, G.; Campolo, M.; et al. Therapeutic Potential of Dimethyl Fumarate in Counteract Oral Squamous Cell Carcinoma Progression by Modulating Apoptosis, Oxidative Stress and Epithelial-Mesenchymal Transition. *Int J Mol Sci* **2023**, *24*, doi:10.3390/ijms24032777.

169. Guarino, V.; Castellone, M.D.; Avilla, E.; Melillo, R.M. Thyroid cancer and inflammation. *Mol Cell Endocrinol* **2010**, *321*, 94-102, doi:10.1016/j.mce.2009.10.003.

170. Nucera, C.; Nehs, M.A.; Mekel, M.; Zhang, X.; Hodin, R.; Lawler, J.; Nose, V.; Parangi, S. A novel orthotopic mouse model of human anaplastic thyroid carcinoma. *Thyroid* **2009**, *19*, 1077-1084, doi:10.1089/thy.2009.0055.

171. Shakib, H.; Rajabi, S.; Dehghan, M.H.; Mashayekhi, F.J.; Safari-Alighiarloo, N.; Hedayati, M. Epithelial-to-mesenchymal transition in thyroid cancer: a comprehensive review. *Endocrine* **2019**, *66*, 435-455, doi:10.1007/s12020-019-02030-8.

172. Zou, M.; Al-Baradie, R.S.; Al-Hindi, H.; Farid, N.R.; Shi, Y. S100A4 (Mts1) gene overexpression is associated with invasion and metastasis of papillary thyroid carcinoma. *Br J Cancer* **2005**, *93*, 1277-1284, doi:10.1038/sj.bjc.6602856.

173. Zou, M.; Famulski, K.S.; Parhar, R.S.; Baitei, E.; Al-Mohanna, F.A.; Farid, N.R.; Shi, Y. Microarray analysis of metastasis-associated gene expression profiling in a murine model of thyroid carcinoma pulmonary metastasis: identification of S100A4 (Mts1) gene overexpression as a poor prognostic marker for thyroid carcinoma. *J Clin Endocrinol Metab* **2004**, *89*, 6146-6154, doi:10.1210/jc.2004-0418.

174. Shi, Y.; Zou, M.; Collison, K.; Baitei, E.Y.; Al-Makhalafi, Z.; Farid, N.R.; Al-Mohanna, F.A. Ribonucleic acid interference targeting S100A4 (Mts1) suppresses tumor growth and metastasis of anaplastic thyroid carcinoma in a mouse model. *J Clin Endocrinol Metab* **2006**, *91*, 2373-2379, doi:10.1210/jc.2006-0155.

175. Salama, I.; Malone, P.S.; Mihaimeed, F.; Jones, J.L. A review of the S100 proteins in cancer. *Eur J Surg Oncol* **2008**, *34*, 357-364, doi:10.1016/j.ejso.2007.04.009.

176. Yang, J.; Barletta, J.A. Anaplastic thyroid carcinoma. *Semin Diagn Pathol* **2020**, *37*, 248-256, doi:10.1053/j.semdp.2020.06.005.

177. Ferrari, S.M.; Elia, G.; Ragusa, F.; Ruffilli, I.; La Motta, C.; Paparo, S.R.; Patrizio, A.; Vita, R.; Benvenga, S.; Materazzi, G.; et al. Novel treatments for anaplastic thyroid carcinoma. *Gland Surg* **2020**, *9*, S28-S42, doi:10.21037/gs.2019.10.18.

178. Holm, T.M.; Yeo, S.; Turner, K.M.; Guan, J.L. Targeting Autophagy in Thyroid Cancer: EMT, Apoptosis, and Cancer Stem Cells. *Front Cell Dev Biol* **2022**, *10*, 821855, doi:10.3389/fcell.2022.821855.

179. Netea-Maier, R.T.; Kluck, V.; Plantinga, T.S.; Smit, J.W. Autophagy in thyroid cancer: present knowledge and future perspectives. *Front Endocrinol (Lausanne)* **2015**, *6*, 22, doi:10.3389/fendo.2015.00022.

180. Abe, I.; Lam, A.K. Anaplastic Thyroid Carcinoma: Current Issues in Genomics and Therapeutics. *Curr Oncol Rep* **2021**, *23*, 31, doi:10.1007/s11912-021-01019-9.

181. Milosevic, Z.; Pesic, M.; Stankovic, T.; Dinic, J.; Milovanovic, Z.; Stojsic, J.; Dzodic, R.; Tanic, N.; Bankovic, J. Targeting RAS-MAPK-ERK and PI3K-AKT-mTOR signal transduction pathways to chemosensitize anaplastic thyroid carcinoma. *Transl Res* **2014**, *164*, 411-423, doi:10.1016/j.trsl.2014.06.005.

182. Wong, K.; Di Cristofano, F.; Ranieri, M.; De Martino, D.; Di Cristofano, A. PI3K/mTOR inhibition potentiates and extends palbociclib activity in anaplastic thyroid cancer. *Endocr Relat Cancer* **2019**, *26*, 425-436, doi:10.1530/ERC-19-0011.

183. Hanly, E.K.; Bednarczyk, R.B.; Tuli, N.Y.; Moscatello, A.L.; Halicka, H.D.; Li, J.; Geliebter, J.; Darzynkiewicz, Z.; Tiwari, R.K. mTOR inhibitors sensitize thyroid cancer cells to cytotoxic effect of vemurafenib. *Oncotarget* **2015**, *6*, 39702-39713, doi:10.18632/oncotarget.4052.

184. Duran, A.; Amanchy, R.; Linares, J.F.; Joshi, J.; Abu-Baker, S.; Porollo, A.; Hansen, M.; Moscat, J.; Diaz-Meco, M.T. p62 is a key regulator of nutrient sensing in the mTORC1 pathway. *Mol Cell* **2011**, *44*, 134-146, doi:10.1016/j.molcel.2011.06.038.

185. Mendoza, M.C.; Er, E.E.; Blenis, J. The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation. *Trends Biochem Sci* **2011**, *36*, 320-328, doi:10.1016/j.tibs.2011.03.006.

186. Ferrari, S.M.; Fallahi, P.; Galdiero, M.R.; Ruffilli, I.; Elia, G.; Ragusa, F.; Paparo, S.R.; Patrizio, A.; Mazzi, V.; Varricchi, G.; et al. Immune and Inflammatory Cells in Thyroid Cancer Microenvironment. *Int J Mol Sci* **2019**, *20*, doi:10.3390/ijms20184413.

187. Carvalho, D.F.G.; Zanetti, B.R.; Miranda, L.; Hassumi-Fukasawa, M.K.; Miranda-Camargo, F.; Crispim, J.C.O.; Soares, E.G. High IL-17 expression is associated with an unfavorable prognosis in thyroid cancer. *Oncol Lett* **2017**, *13*, 1925-1931, doi:10.3892/ol.2017.5638.

188. Rong, Z.; Wang, A.; Li, Z.; Ren, Y.; Cheng, L.; Li, Y.; Wang, Y.; Ren, F.; Zhang, X.; Hu, J.; et al. IL-17RD (Sef or IL-17RLM) interacts with IL-17 receptor and mediates IL-17 signaling. *Cell Res* **2009**, *19*, 208-215, doi:10.1038/cr.2008.320.

La borsa di dottorato è stata cofinanziata con risorse del
Programma Operativo Nazionale Ricerca e Innovazione 2014-2020 (CCI 2014IT16M2OP005),
Fondo Sociale Europeo, Azione I.1 "Dottorati Innovativi con caratterizzazione Industriale"