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THE COMPLEX RELATIONSHIP BETWEEN CHRONIC INFLAMMATION AND DIFFERENTIATED THYROID CANCER: EVIDENCE FROM *IN VITRO* AND CLINICAL STUDIES

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SUMMARY

ABSTRACT 3
Introduction 5
Differentiated Thyroid Cancer 5
Clinical characteristics of Differentiated Thyroid Cancer 5
Epidemiology of thyroid cancer 8
Diagnostic work-up of Differentiated Thyroid Cancer (DTC) 9
Therapeutic options for DTC 10
Management of advanced/metastatic DTC 11
The tumor microenvironment 12
Chemokines 13
Chemokines in the DTC microenvironment 14
Pro-tumorigenic chemokines – the pivotal role of CXCL8 15
CXCL8 and DTC 17
CXCL8 as a target of anti-cancer therapies 18
Chronic Autoimmune Thyroiditis 19
Chronic Autoimmune Thyroiditis and Thyroid Cancer 21
AIM OF THE THESIS 23
Materials and methods 26
AIM 1 26
AIM 2 30
Results 32
AIM 1 32
AIM 2 37
Discussion 39
Figures 46
Figure Legends 56
Tables 62
REFERENCES 64

ABSTRACT

Inflammation is an essential component of malignancies, including differentiated thyroid cancer (DTC). Thyroid cancer microenvironment is composed of a mixture of immune cells and soluble mediators. Among them, the chemokine CXCL8 exerts multiple pro-tumorigenic activities, including a chemotactic action on circulating neutrophils, induction of tumor cells growth, increase in angiogenesis and induction of the epithelial to mesenchymal transition, which promotes cell migration. Clinical studies in patients affected by several types of cancer evidenced that CXCL8 serum levels reflect the tumor burden and are related with the tumor aggressiveness. Solid evidence indicates that CXCL8 targeting can reduce tumor progression. The controversial relationship between inflammation and thyroid cancer tumorigenesis involves also the debated topic of the association between chronic-autoimmune-thyroiditis (CAT) and (DTC). DTCs are often diagnosed in the context of CAT and display an inflammatory-immune cells infiltration at histology, but whether the malignant transformation is promoted by the inflammatory response, or the peri-tumoral inflammation is induced by cancer-specific inflammatory molecules is still a matter of debate. This thesis project had two principal aims, i) to investigate the role of a pro-tumorigenic chemokine (CXCL8) in thyroid cancer microenvironment and to test *in vitro* the modulating properties of two different pharmacologic agents (PLX4720 and phenformin) and ii) to evaluate if CAT is a risk factor for the de novo development of DTC through a longitudinal population study. For Aim 1, thyroid cancer cell lines both BRAFV600E mutated (BCPAP, 8305C, 8505C) and RET/PTC rearranged (TPC-1), and normal human thyrocytes (NHT) were cultured alone or after treatment with two PLX4720 ore phenformin at increasing concentrations. CXCL8 concentrations were measured in the cell supernatants. Cell viability was evaluated through WST-1 and Annexin/propidium assay. Metastatic potential was assessed with migration assay and colony formation assay. For Aim 2, a retrospective longitudinal cohort study was designed including 510 CAT patients with a 10-years follow-up. The results of the first part of the thesis demonstrate that thyroid cancer cells secrete high amounts of CXCL8 and that both PLX4720 and phenformin are able to exert several anti-cancer activities within

cancer microenvironment that are in part due to the inhibition of CXCL8 secretion. The results of the second part of the study indicate that the presence of CAT is not a risk factor for the new onset of DTC during long-term follow-up. The results of this thesis project suggest that two different kinds of inflammation (cancer-related and autoimmunity-related) exert different effects on DTC microenvironment and could have opposite effects on DTC development and prognosis.

INTRODUCTION

Differentiated Thyroid Cancer

Clinical characteristics of Differentiated Thyroid Cancer

Thyroid cancer (TC) accounts for approximately 3% of all human cancers and has an incidence of approximately 7.5-8 cases per 100,000 individuals for men and approximately 22 new cases per 100,000 individuals for women, with a mortality rate of 0. 5 out of 100,000 for both genders. It is the most frequent endocrine neoplasm, however its prevalence, among all malignant tumors in humans, is relatively low (less than 1% of all malignant neoplasms), despite the fact that benign nodules are extremely frequent in the general population (1).

Sex is commonly considered a risk factor for thyroid cancer, since women are 3 to 4 times more frequently affected than men, although men experience a more aggressive disease (2). Other risk factors include a family history of TC, exposure to radiation and flame retardants and smoke (3).

The histotypes of TC are classified according to the originating cell type: papillary thyroid cancer (PTC) (≈80% of cases) and follicular thyroid cancer (FTC) (≈2%) arise from differentiated follicular cells, medullary thyroid cancer (TCM) (≈4%) originates from parafollicular cells. More rarely TC can present as a poorly differentiated TC (PDTC) or anaplastic thyroid carcinoma (ATC) (≈5-10%), which are the cause of the majority of deaths from thyroid cancer(4). For the purpose of this thesis, we will focus only on thyroid cancer originating from follicular cells.

Papillary thyroid cancer (PTC) is the most frequent of follicular cell-derived tumors. Its histological characteristics include high density of nuclei that tend to overlap and have grooves, irregularities of the nuclear membranes, presence of intranuclear inclusions of cytoplasm and eccentric nucleoli, enlargement, and elongation and “ground glass” appearance of nuclei (" Orphan Annie eyes ") (5). Some histological variants can be more aggressive, the columnar, tall cell, solid and hobnail variants (6). Most PTCs occur in adults 20 to 50 years of age, although it can also be found in children and the elderly, and there is a female predominance, especially in the fourth decade of age, with a 4: 1

female/male ratio (7). PTC tend to extend to the soft tissues of the neck or to latero-cervical lymph nodes, which sometimes constitute the first clinical manifestation of the disease. On the other hand, long-distance metastases to organs such as lung, bone, central nervous system, pancreas and breast are rare. The prognosis for papillary carcinoma is excellent, with a 10-year survival of over 90% of patients.(5)

Follicular thyroid cancer (FTC) is the second in frequency of occurrence among thyroid carcinomas and shows a higher incidence (20–40%) in geographic areas where iodine deficiency is detected (8). Is more common among women between the fifth to sixth decade of life. The diagnosis of follicular carcinoma is based on the histopathological demonstration of the infiltrative growth of the tumor, which allows classifying FTCs into widely invasive and minimally invasive forms. While largely invasive FTC are usually large masses with local extension to soft tissues and vessels, with a worse prognosis, minimally invasive FTCs usually present as a well-defined, smaller mass. Most FTCs have different foci of capsular or vascular invasion and tend to spread haematogenically with metastases to the brain, bone, liver and lungs. FTC shows a good prognosis which however depends on the degree of invasion.

According to the most recent WHO guidelines, encapsulated non-invasive follicular PTC variants are now referred to as NIFTPs ('non-invasive follicular thyroid neoplasm with papillary-like nuclear features'). This nomenclature changes, which eliminates the term "carcinoma", is based on the clinical evidence of their less aggressive behaviour during long-term follow-up, and shjould hopefully lead to a reduction in the unnecessary use of total thyroidectomy and radioactive iodine (RAI)(9).

Scarcely Differentiated Thyroid Cancer and Anaplastic thyroid cancer (ATC) are both derived from a de-differentiation of follicular-cell-derived cancers and are responsible for more than half of all deaths attributable to thyroid cancer in the United States. These cancers usually present as large, fast-growing masses composed by small neoplastic cells with hyperchromatic nuclei and high mitotic index. ATC incidence is about 1-2 cases / million, mainly in elderly individuals, with a female: male

ratio of 1.5: 1(4). They tend to invade surrounding soft tissues such as the trachea, pharynx, esophagus and regional lymph nodes and lung, bone and brain metastases can be found in 10-40% of patients. The prognosis of anaplastic carcinoma is extremely poor, with a mean survival of 2 to 7 months and a 5-year survival of 0-14%(4).

DTC can harbor three main types of genetic mutation:

- BRAF mutations: BRAF is a serine-threonine kinase belonging to the RAF family of proteins, which plays a mediator role in the MAPK signaling pathway. About 45% of papillary carcinomas (less frequently in the follicular variant) and about 30% of anaplastic carcinomas carry a mutations in the BRAF gene. The most frequent mutation is the amino acid substitution valine-glutamate on the residue 60014.15, which causes the constitutive activation of BRAF and the MAPK "pathway". Another consequence of this mutation is an altered functioning of the iodine-sodium symport (NIS) and in general of the genes that control iodine metabolism; this could explain the poor response to radioiodine therapy (10,11).

- RET / PTC rearrangements: RET is a proto-oncogene encoding a membrane receptor tyrosine kinase; this can be constitutively activated in the follicular cells through a gene rearrangement, most frequently the RET /PTC 1 and RET / PTC 3, leading to a hyper-activation of the MAPK "pathway". RET / PTC rearrangement is typical of PTC (up to 20%) and is a risk factor for lymph node spread (12,13)

- RAS: the RAS genes (H-RAS, K-RAS, N-RAS) are involved in the regulation of a very large number of intracellular signaling pathways and their de-regulation is involved in the pathogenesis of different types of cancers. About 50% of follicular carcinomas, 50% of anaplastic carcinomas and 20% of papillary carcinomas (follicular variant only) are characterized by mutations affecting codon 61 of N-RAS and H-RAS(14).

Epidemiology of thyroid cancer

The incidence of thyroid cancer rapidly increased during the second half of the 20th century in the United States and in other developed countries, while mortality remained relatively constant(15). This increase was found mainly in small (<2 cm) papillary cancers, with an absolute increase in the rate of thyroid cancer in women four times higher than in men.

The explanations for this worldwide increase are controversial. Indeed, a large portion of the increased incidence of thyroid cancer is probably due to an increase in diagnostic procedures, such as neck ultrasounds, that leads to the detection of small thyroid nodules that would be missed at physical examination. The resulting overdiagnosis has been accompanied by overtreatment with an excessive number of surgical intervention and radioiodine therapy(16). Moreover, an increase in the number of surgical interventions for non-malignant thyroid diseases (such as Graves' disease an Multinodular Goiter) has increased the detection of incidental malignant tumors(17,18).

This trend reached its peak of 15.2 per 100,000 individuals in 2013, then a gradual decline was observed. In particular, the last 2021 SEER report showed that the 5-year incidence trend for thyroid cancer is statistically significantly decreasing among women and is stable among men(1). The observed declines in overall thyroid cancer incidence are likely attributable to changes in diagnostic practices for low risk tumors suggested by the most recent guidelines (19) and, to a lesser extent, to diagnostic coding changes for follicular variant of papillary thyroid carcinoma (20).

Nevertheless, the observed increased incidence of more advanced-stage cancers (1) suggests that improved detection techniques are not the only reason for this phenomenon.

The following possible risk factors have been proposed:

- Exposure to ionizing radiation, particularly at young age, due to diagnostic procedures or radiotherapy (21,22).

- Exposure to environmental pollutants such as asbestos, benzene, formaldehyde, pesticides, bisphenol A (BPA), polychlorinated diphenyls (PCBs) and polyhalogenated aromatic hydrocarbons (PHAH), which can act as genotoxic or non-genotoxic carcinogens(23). The volcanic environment also appears to increase the incidence of thyroid cancer (24).
- Obesity: some authors suggest a causal link between the obesity epidemic worldwide and the increased incidence of thyroid cancer(25), although this association is still controversial(26).

Diagnostic work-up of Differentiated Thyroid Cancer (DTC)

The diagnostic work-up of DTCs includes both pre- and postoperative pathological and molecular assessments. Thyroid Ultrasound (US) is the most used technique to stratify the risk of malignancy of thyroid nodule, due to its low invasiveness and efficacy. Nevertheless, US is a user-dependent technique, and results are not always easy to compare. With this purpose some systems of classification of ultrasonographic risk were created, among which the EU-TIRADS and ACR-TIRADS are the most used, respectively in Europe and America(27,28).

Both TIRADS systems (*Thyroid Imaging and Reporting Data System)* classify thyroid nodules in five different categories of increasing risk, according to their ultrasonographic characteristics; each of these categories is associated with a different malignancy risk.

These systems provide essential operational guidelines about the management of thyroid nodules: they help to select those which should be subjected to FNAB and, in the case where this is not indicated, they suggest the frequency of the ultrasonographic follow-up (29,30).

Preoperative Fine-Needle-Aspiration-Cytology (FNAC) is the main tool to evaluate the risk of malignancy of a thyroid nodule. The decision to perform FNAC should be based on lesion size and ultrasonographic characteristics. Cytology findings are classified into diagnostic categories associated with different risks of malignancy, according to the specific classification method employed (31). Most malignant thyroid tumors can be identified cytologically, with the exceptions

of FTCs and the newly defined 'non-invasive follicular thyroid neoplasm with papillary-like nuclear features' (NIFTP), which are usually classified as indeterminate in the various thyroid cytology reporting schemes(32,33). FNA-based diagnosis of poorly differentiated carcinoma is also challenging unless there is an obviously increased mitotic activity and/or necrosis.

FNA diagnosis can be facilitated by assessment of malignancy markers (including proteins commonly overexpressed in tumors, e.g.HBME1 or galectin-3) and molecular alterations specifically associated with malignancy (e.g. BRAF mutations, RET fusions, other novel gene alterations). Specifically designed gene panels are reportedly useful for identifying malignancy when cytology samples are morphologically indeterminate (33).

Therapeutic options for DTC

While well-differentiated thyroid tumors (papillary and follicular carcinoma) are highly treatable and generally curable; poorly differentiated and undifferentiated tumors (anaplastic carcinoma) are less common, more aggressive, metastasize early and have a poor prognosis.

Primary tumour management is determined by the results of a preoperative risk assessment. In case of low-risk, unifocal papillary microcarcinomas (<1 cm), active yearly US surveillance of the thyroid and neck lymph nodes can be proposed (34). Surgery is the therapy of choice in all other cases and has the aim of completely removing the tumor. The procedure may consist of a total thyroidectomy or a lobectomy, based on the age of the patient and the size of the nodule. Total thyroidectomy has the advantage of eliminating also contralateral foci of the tumor and of reducing the risk of dedifferentiation of any residual tumor towards an anaplastic cell type. Nevertheless, the risk of recurrent laryngeal nerve injury and temporary or permanent hypoparathyroidism, even in the hand of expert surgeons, is not negligible(35). Lobectomy, on the other hand, can be considered in cases of small (<1 cm), low-risk and unifocal papillary carcinomas, in the absence of previous irradiation of the head and neck or of lymph node cervical metastases(36). Also in cases of anaplastic thyroid

cancer, a total thyroidectomy allows to reduce the compressive symptoms that can be caused by the tumor(37).

Radioactive iodine therapy (RAI) is a therapeutic option for patients undergoing total thyroidectomy. RAI can be used to eliminate the normal thyroid remnant, thereby ensuring undetectable serum Tg levels (in the absence of neoplastic tissue), which facilitate follow-up (remnant ablation); to irradiate presumed foci of neoplastic cells, thereby reducing the recurrence risk (adjuvant therapy); and/or treat persistent or recurrent disease (treatment of known disease)(38).

RAI can be administered in conjunction with two possible thyroid stimulating hormone (TSH / thyrotropin) stimulation methods, suspension of thyroid hormone or administration of recombinant human thyrotropin (rhTSH).

While practice guidelines unanimously recommend treatment with high RAI activities (>100 mCi) for patients with high risk of recurrence, RAI is not recommended for patients with 'very low-risk' of recurrence (39,40). On the other hand, there is less consensus regarding patients with low or intermediate risk of recurrence. According to the recent statement by several scientific societies, the decisions to treat should be taken on an individual basis, depending on tumor features, health-care setting (e.g. availability and quality of thyroid surgeons, US, RAI imaging, Tg assays), patient-related factors (e.g. comorbidities, motivation, emotional concerns), and the local management team's preferences (41).

After thyroid surgery, except for lobectomy, patients undergo thyroid replacement therapy with levothyroxine with the aim of reaching a target TSH level according to the risk of relapse and the patient's co-morbidities (42).

Management of advanced/metastatic DTC

Less than 10% of DTC patients experience distant metastases, half of them at diagnosis and the other half after initial treatment. Metastases are more frequent in cases of large tumors, with aggressive

histological subtypes, vascular invasion, macroscopic extrathyroidal extension and local lymph node involvement (19). The most common sites are bones and lungs (in 25% and 49% of cases, respectively). Bone metastases are more common in FTC than in PTC (55.5% versus 31.5%, respectively). The presence of distant metastasis greatly increases the 5-years and 10-years mortality rate (65% and 75%, respectively)(43,44). The available therapeutic options include repeated RAI therapy (as long as the tumor does not become RAI-refractory), locoregional therapy, such as External Beam Radiation Therapy (EBRT), local surgery or percutaneous ablation (45-47), and systemic therapy. Among the targeted drugs that are approved for metastatic DTC, lenvatinib and sorafenib, both anti-angiogenetic agents, are considered as first-lines (48,49). These drugs should be initiated only after demonstration of radiological progression in RAI-refractory cancers. Other drugs are being studied as curative agents for metastatic DTC, including:

- BRAF-inhibitors and MEK-ERK inhibitors: this class of drugs includes Sorafenib(50), Vemurafenib (or PLX4720) (51,52) and Dabrafenib (53,54)
- Larotrectinib for cancers expressing the TRK gene fusion protein(55)
- Checkpoint inhibitors (56).
- AMPK-inhibitors, including AICAR, metformin and phenformin (57,58)

Systemic therapy is used only for the treatment of anaplastic thyroid carcinoma, which does not respond to therapy with 131 I and for which the use of single anticancer drugs induces only partial remissions in some patients. (59)

The tumor microenvironment

The process of formation of a neoplasm, or tumorigenesis, is a complex and dynamic phenomenon that consists in the acquisition of peculiar characteristics, responsible for the proliferation of cells and the spread of the tumor, which consist of: increase in proliferative signals, evasion from the mechanisms that negatively regulate growth, resistance to apoptosis, unlimited replication,

stimulation of angiogenic phenomena, tendency to local invasion and formation of metastases and evasion from the immune response(60).

The tumor grows and expands around a cellular and extracellular environment which is commonly referred to as a tumor microenvironment. Several studies on cancer patients have shown that the local profile of the tumor microenvironment is closely related to its biological behavior and that its composition can also influence the patient's therapeutic outcome(61,62). The tumor microenvironment consists of proliferating neoplastic cells, stromal cells, which include fibroblasts, vessel cells (endothelial cells, pericytes, smooth muscle cells), blood vessels, infiltrating inflammatory cells and a variety of associated tissue cells. Immune cells that constitute the tumor microenvironment include T lymphocytes, dendritic cells (DCs), B cells, macrophages, polymorphonuclear leukocytes and rare natural killer (NK) cells(63). The recruitment of these cells, alone or in combination, is regulated by the presence of specific proteins called chemokines(64).

Chemokines

Chemokines are small molecules secreted both by normal and cancerous cells within the tumor parenchyma. Their action is exerted through the bind which have receptors on their surface capable of recognizing and binding them, thus allowing their action. These are soluble proteins are secreted within the extracellular matrix (ECM), establishing a concentration gradient able to induce chemotaxis of cells expressing specific chemokine G-couples receptors (65,66). Chemokines have a low molecular weight and can be divided into four classes according to the position of the first two cysteine residues (C) in their protein sequence: CC chemokines, CXC chemokines, C chemokines and CX 3C chemokines(67). They can also be divided into inflammatory chemokines, induced by acute or chronic inflammatory processes or by adaptive immunity responses, and homeostatic chemokines, constitutively expressed in specific tissues or cells(68).

More than 50 different chemokines have been described in literature. The chemokine-chemokine receptor binding system is redundant, since there are more chemokines than receptors, meaning that many receptors bind to more chemokines and vice versa (65).

Within the tumor microenvironment, chemokines, besides the ability to attract and maintain immune cells at the tumor site, induce various pro-tumorigenic actions, which include proangiogenic, cytoproliferative and pro-metastatic effects (69). The inflammatory process, and in particular chemokines, therefore have a key role within the tumor microenvironment, where the body's normal defense mechanisms can counteract of favor the processes of carcinogenesis and neoplastic progression(70).

The binding of a chemokine to its G-coupled receptor induces a series of downstream effects leading to the activation of several intracellular pathways(71). The two main responses are the activation of integrins, which cause cell adhesion, and the polymerization of actin, leading the cell to orientate in space and polarize. This process, in association with the contraction of the actomyosin induces the cell to migrate. The relevant role of chemokines in cancer biology is also linked to their ability to activate other signaling systems, such as the tyrosine kinase receptor pathway and the JAK-STAT pathway(70). Some chemokine receptors are labeled as "atypical", since they share the 7-domain transmembrane structure of conventional receptors but are not coupled to G proteins. Consequently, they fail to induce classical receptor signaling and downstream intracellular responses but act as scavengers, which target chemokines for lysosomal degradation(65).

Chemokines in the DTC microenvironment

Differentiated Thyroid carcinoma is characterized by a complex inflammatory microenvironment in which immune cells like macrophages, T lymphocytes, dendritic cells (DC) and natural killer (NK) cells can be identified, circulating between the neoplastic cells and the supporting stroma, and which can represent up to 50% of the tumor mass(72). The metastatic potential of DTC is in part regulated by the interaction of malignant thyroid cells with the chemokine system present in the tumor

microenvironment(73). Thyroid cells produce a broad spectrum of CXC chemokines (eg, CXCL1, CXCL8, CXCL9, CXCL10 and CXCL11) under basal conditions and / or under the influence of specific stimuli(74). In thyroid tissue, T helper 1 (Th1) lymphocytes, recruited for the inflammatory response, can stimulate the production of IFN-γ and tumor necrosis factor α (TNF-α), which in turn induce an increase in secretion of chemokines by thyroid cells. An amplification loop is thus generated, activating and perpetuating the autoimmune process(75). The RET / PTC1 rearrangement (76) and the V600E point mutation in the BRAF gene (77) also contribute to the induction of a protumorigenic inflammatory microenvironment through the activation of their respective proto-oncogenes. The RET / PTC1 oncogene induces the expression of a proinflammatory program that includes the upregulation of several chemokines such as CCL2, CCL20, CXCL8, CXCL1, CXCL10 and CXCL12 and the chemokine receptor CXCR4, along with other proinflammatory cytokines and enzymes(12,76,78). The BRAFV600E mutation, on the other hand, is strongly correlated with an overexpression of CXCR4 (79) and an increase in the secretion of several pro-inflammatory mediators, including CXCL8(54,80)

Pro-tumorigenic chemokines – the pivotal role of CXCL8

Several chemokines, including CCL15, CXCL12, CXCL16, CXCL1, CCL20 and CCL2 have pro-tumorigenic effects on thyroid cells (81). Nevertheless, CXCL8 appears to have a pivotal role in thyroid cancer microenvironment. At present, CXCL8 is probably the most studied chemokine in human cancer(82). The discovery of CXCL8, often referred to as interleukin-8 or chemotactic factor / activating neutrophils-1 (83,84) has represented a landmark in immunology as it is the first identified immune molecule whose chemotactic activity was demonstrated on selected subpopulations of leukocytes, both at the sites of inflammation and tissue injury (85). Cancer cells secrete CXCL8 and create a concentration gradient in the tumor microenvironment that attracts neutrophils, which express the CXCR1 and CXCR2 receptors and, once in place, release enzymes responsible for remodeling the extracellular matrix; this effect facilitates the migration of tumor cells and their entry into the

vascular bed, allowing them to travel to metastatic sites(86). Beside this, CXCL8 has a broad spectrum of actions, including the activation of cells expressing CXCR1 / CXCR2 (87).

The expression of the CXCL8 gene and subsequent secretion of the corresponding protein are mainly regulated by the nuclear transcription factor NF-κB and activator protein-1 (AP1), but other, yet to be elucidated, pathways are also thought to be involved (88).

CXCL8 in normal tissues is expressed at low or undetectable levels, as it is linked to proinflammatory states, but its expression increases in several human solid tumors such as melanoma (89), squamous cell carcinoma(90), cervical carcinoma(91), ovarian cancer(92), non-small cell lung cancer (93), colon cancer (94)and gastric cancers (95). The widespread expression of CXCL8 in human neoplasms is of particular relevance, since the pleiotropic effects of this chemokine influence cell growth, angiogenesis, invasiveness and metastatic potential(81). The secretion of CXCL8 by tumor cells can improve their proliferation and survival rate through autocrine signaling pathways by binding to its G-coupled receptor and activation of the mitogen-activated protein kinases (MAPK). These processes lead in the end to an increase in cell proliferation and survival (96-99).

CXCL8 is also potent pro-angiogenic factor, since it induces the formation of new blood vessels from pre-existing capillaries, which supply and nourish the tumor cells, thus allowing the growth of the solid tumor The angiogenic effect of CXCL8 occurs after its binding to the CXCR1 and CXCR2 receptors expressed by endothelial cells (100), which causes their activation, their chemotaxis (as demonstrated by in vitro experiments) and the formation of new vessels blood in vivo (101,102).

Most importantly, CXCL8 has significant pro-metastatic properties (103,104) due to its ability of recruiting neutrophils to the tumor site that, in turn, favors the migration of neoplastic cells (105). CXCL8 is also involved in the epithelium-mesenchymal transition (EMT), a molecular process that leads epithelial cells to lose some of their typical characteristics (e.g. apical-basolateral polarity, extensive intercellular adhesion and contact inhibition of growth) and to acquire the typical characteristics of mesenchymal cells, including edge asymmetry, loss of intercellular contact, and

increased motility and invasiveness(106,107). Since also EMT itself is able to promote the secretion of CXCL8, a positive loop of pro-tumorigenic effects can be established(108).

CXCL8 has been also used as a tumor biomarker. Indeed, CXCL8 serum levels are higher in several cancer patients (including lung cancer, melanoma, renal cell carcinoma) when compared to healthy controls. Moreover, CXCL8 detected both in sera and cancer tissue are related with tumor size, depth of infiltration, stage and the prognosis, and low CXCL8 serum levels predict a less aggressive course of and a better response to anticancer therapy(109,110).

CXCL8 and DTC

The link between CXCL8 and thyroid was discovered in 1992, when it was first demonstrated that normal thyroid cells in primary cultures secrete CXCL8 (111). More recent studies have also revealed that iodide positively regulates the expression of the CXCL8 gene(112), suggesting that this chemokine has a role in thyroid homeostasis(113).

CXCL8 is released in higher quantities by cells derived from papillary, anaplastic and poorly differentiated thyroid cancers (80,114,115).The pattern of secretion of CXCL8 has been thoroughly characterized in different kinds of normal and cancerous thyroid cell lines *in vitro*. First of all, early reports showed that the presence of RET-rearrangement in PTC samples leads to an overexpression of the gene encoding for CXCL8 (12). Moreover, the concentration of CXCL8 in cell supernatants significantly differs between different cell lines, both at baseline and after TNF-α stimulation(116). BCPAP cells (bearing BRAF-V600E mutation) show a higher level of basal secretion of CXCL8 when compared with TPC-1 cells (bearing the RET / PTC1 translocation), but the treatment with TNF-α induces an higher degree of stimulation of CXCL8 secretion in TPC-1 cells that in BCPAP(116)..

As for its pro-tumorigenic effect, is CXCL8 promotes the metastatic process of thyroid neoplastic cells: and increase in the migration ability was demonstrated *in vitro* (117)and increases metastatic

spread, with a consequent increase in mortality (118). Taken together, the results of in vitro and in vivo studies therefore provide evidence of an action of CXCL8 as a pro-tumorigenic agent and suggest that reducing chemokine levels in the thyroid tumor microenvironment could be of therapeutic benefit.

CXCL8 as a target of anti-cancer therapies

Having established the ability of the chemokines secreted within the tumor microenvironment to promote neoplastic progression, the concept of reducing their secretion by cancerous and non-cancerous cells of the TME seems a viable therapeutic option for anti-cancer therapies. The possibility of combining chemokine-based therapies with current tumor immunotherapies is also under discussion(119). Chemokines are able to control the immune cell trafficking into the tumoral mass, which are essential for obtaining an effective immune response induced by immunotherapic agents. The entry of immune cells into the tumor can be favored by therapies that modulate the secretion of chemokines in an anti-tumorigenic way (120). Possible approaches for interfering with chemokine secretion include neutralizing and monoclonal antibodies, small molecules, modified chemokines, proteins with binding domains and pharmacological modulation of chemokines / chemokine-receptor interaction (121,122); the application of these therapeutic agents was successful in the treatment of some types of cancer, including mouse models of breast, lung and ovarian cancer, where the targeting of CCL2, CCL3 or CCL5 signaling pathways was able to inhibit metastatic spread and cancer angiogenesis (123,124).

The CXCL8-CXCR1/2 axis is also an important target for immunotherapy [211], and any strategy that aims at interfering with this system may profoundly influence the nature of tumor microenvironment(125). A number of small CXCR1/2 inhibitors, CXCL8 release inhibitors and neutralizing antibodies to CXCL8 or CXCR1/2 have been developed over the past two decades. These include Repertaxin (also known as Reparixin), a non-competitive allosteric inhibitor of CXCR1 and CXCR2 originally developed to block CXCL8 activity and reduce tissue damage after myocardial

infarction or stroke (119,126). The experimental inhibition of CXCL8 secretion by thyroid cancer cells with different therapeutic agents (such as interferons, Metformin or AMPK-inhibitors) leads to important results, including inhibition of cell migration in *vitro* (127-129) and inhibition of the metastatic process in animal models with thyroid cancer, increasing their survival rate(118). Finally, the use of a CXCR4 antagonist revealed an antitumor effect in a PTC cell line, given by the inhibition of the migration of thyroid cancer cells at low concentrations and by the inhibition of proliferation at high doses(130).

Although the results obtained in the studies on chemokines and their receptors are to be considered as preliminary, it is reasonable to hypothesize that the development of immunotherapy strategies aimed at targeting these proteins or their signaling in thyroid cancer may be promising (65).

Chronic Autoimmune Thyroiditis

Chronic autoimmune thyroiditis (CAT) is the most common autoimmune thyroid disease and the most common cause of hypothyroidism. The pathogenesis of CAT is due to the breakdown of immune tolerance towards the thyroid gland. Following an initial stimulus, such as environmental factors including iodine intake, among others, formerly thyroid-tolerant immune cells become activated and lose their thyroid tolerance. As a consequence, leukocytes s infiltrate the tissue and promote an autoimmune response. Under an anatomopathologic point of view, CAT is characterized by diffuse lymphocyte infiltration, fibrosis, and parenchymal atrophy. It is also associated with thyroid dysfunction and, occasionally, the development of goiter(131,132). Ninety percent of the cases have detectable circulating anti-thyroid peroxidase (Ab-TPO) and anti-thyroglobulin (Ab-Tg) antibodies, although the disease can occur also in serum-negative patients(133). Sonographic findings of CAT include decreased echogenicity, heterogeneity, hypervascularity, and presence of hypoechoic pseudo-nodules with echogenic rim. CAT is a histological diagnosis by strict criteria, but an histologic sample is not required for diagnosis, which can be made by clinical evaluation and antibody measurement.

The disease occurs in .3–1.5 per 1000 individuals worldwide and is more predominant in females with gender prevalence ratios of 5 to 20:1(134).

The immune mechanisms underlying the pathogenesis of CAT are several and can be summarized as follows:

- Target antigens: Thyreoperoxidase (TPO) and Thyroglobulin (Tg) are the two main auto-antigens for the autoreactive T-cells that can be found in sera and thyroid infiltrates of patients with CAT(135-137), as also confirmed by mouse models with thyroiditis (138,139)

- Cellular immune response: CAT is characterized by intrathyroidal accumulation of leukocytes induced by the secretion of cytokines and chemokines that commonly belong to the T helper 1 (Th1) immune response. The process probably begins with the infiltration of antigen-presenting-cells, like dendritic cells, which activate intrathyroidal lymphocytes(140). A dysfunction in regulatory T-cells is probably required to overcome peripheral tolerance(141)

- Cytokine/chemokine milieu: an imbalance in the amount of chemokines and cytokines towards a pro-inflammatory environment [e.g., interferon-g (IFN-g) in first line, IFN-a, tumor necrosis factor-alpha (TNF-a)] is observed in thyroid tissue samples and the peripheral blood of CAT patients, leading to thyroid tissue damage (142). On the contrary, lower amounts of the anti-inflammatory cytokine tumor growth factor-b1 (TGF-b1) contribute to disease progression as a result of decreased immunosuppressive effect (143). Remarkably, thyrocytes themselves (HT-derived or stimulated) can become a source of cytokine and chemokines and are directly involved in the immunological process as both target and immune influencing cells (74,144). In particular, the IFN-induced chemokine CXCL10 has a pivotal role in enstablishing and maintaining the autoimmune process (75,145).

- Humoral immune response: circulating antibodies recognizing TPO and Tg specific antigens are highly prevalent in hypothyroid patients and their presence in euthyroid patients predicts a progression towards hypothyroidism during follow-up (146,147). Nevertheless, in up to 20% of

hypothyroid patients thyroid autoantibodies are not detectable, indicating the pivotal role for the cellular immune response that seems to occur before the humoral immune response(148).

Chronic Autoimmune Thyroiditis and Thyroid Cancer

The association between Chronic Autoimmune Thyroiditis (CAT) and DTC was first proposed by Dailey et al in 1955 (149). The link between these two disorders is appealing because the concept of chronic inflammation leading to a neoplastic condition is well established in other contexts (70,150), but the debate on the issue is still ongoing. DTCs are frequently diagnosed in patients with a diagnosis of CAT and, at histology, the tumor masses display an inflammatory-immune cell infiltration (151,152). However, whether CAT is the responsible for the malignant transformation or cancer-specific inflammatory molecules induce the peri-tumoral immune infiltrate is still a debated topic. Several studies investigated this association between CAT and DTC, obtaining discordant findings. A clear association between CAT and DTC, mainly papillary, was observed in several surgical series (153-161), whereas such an association was not found in cytological studies (162-169). These findings were also confirmed by a recent meta-analysis that showed a DTC prevalence of 1.20 % (RR = 0.69) in CAT patients from cytological series, while it increased to 27.56% (RR = 1.59) in archival thyroidectomy series (170).

The reasons for this discrepancy may be due to the fact that early surgical series carry the risk of selection biases. First of all, thyroidectomy is not usually required in patients with CAT, so the indication for the procedure can be found, especially in early series, in the presence of a suspicious nodule, or of the uncommon occurrence of huge Hashimoto's goiters producing compression, or of historic or physical findings warranting further workup and treatment (e.g., irradiation, nerve paralysis, pain, or cervical lymph node enlargement)(171). Consequently, CAT patients who require thyroidectomy would already be at higher risk for malignancy compared to the general population with CAT, leading to a selection bias (164).

Moreover, the fact that most PTC are slowly growing and asymptomatic malignancies, and that their population incidence increases sharply whenever screening procedures are implemented, further suggests that a selection bias may impair the results of surgical series (18,172). Indeed, patients with CAT are more likely than the general population to undergo thyroid US, both at diagnosis and during the follow-up. As a partial confirmation of this hypothesis, Jackson et al, recently reported that CAT is a risk factor especially for incidental thyroid cancers, and not clinically overt ones (173).

To the best of our knowledge, only one study tried to assess longitudinally the incidence of DTC in CAT patients, and its results failed to show any significant differences between more than 800 patients with CAT and a group of age and sex-matched controls in terms of DTC incidence. However, as at the time of the study thyroid ultrasound was not routinely performed, small asymptomatic cancers may have been missed, and the absence of thyroid nodules at baseline may only be postulated(174).

Other studies investigated whether the presence of CAT could influence the clinical course and the prognosis of DTC. DTC with concurrent CAT is associated with younger age and female gender, but also with less aggressive features, including small tumor size, less frequent capsular invasion and nodal metastasis, and better prognosis (162,175-177) and, in the end, a lower recurrence probability and higher survival rate (178). Finally, the coexistence of CAT is significantly associated with a lower frequency of BRAF mutations (167,179).

The possible explanation of this better prognosis is that the lymphocytic infiltrate of typical of CAT could have a cancer-retarding effect, contributing to a favorable outcome of DTC. Intriguingly, both CAT and DTC are characterized by an immune infiltrate(131), but its qualitative characteristics may be the key element that influences DTC prognosis.

Indeed, while some studies showed that the presence of an immunological infiltrate of T cells, B cells, macrophages, and Th17 cells within DTC microenvironment is related with a better prognosis (180), other authors observe a correlation between leukocyte infiltrate (in particular of tumor-associated T

cells and macrophages and Tregs) within the tumor and an increased risk of tumor progression and invasion, lymph node metastasis and cancer-related mortality (181-183).

In summary, the presence of a tumor microenvironment rich in pro-tumorigenic elements would favor cancer progression and metastasis. These elements include components of the cellular infiltrate (such as Regulatory T-cells and Mesenchymal Derived Stromal Cells) and soluble elements (including pro-tumorigenic chemokines like CXCL8) that can exert direct pro-tumorigenic activities but also impair and silence the host immune response against the tumor.

On the other hand, a tumor micro-environment characterized by an immunological infiltrate rich in cytotoxic CD8+ cells and dendritic cells and with a high level of anti-tumoral chemokines (such as CXCL10) would induce a more benign course of the disease and favor the controlling effect of the host immune response against the tumor. The high concentrations of CXCL10 and the immunological infiltrate rich in dendritic cells typical of CAT would exert a tumor-controlling effect on the cancerous mass.

This hypothesis is in part confirmed by a recent work by Pani et al: the authors created a mouse model in which DTC and CAT develop in a predictable manner, showing that the tumor phenotype profoundly differs according to the timing of CAT development (preceding as opposed to coinciding with the onset of DTC). Mice with a pre-existing CAT experienced a less aggressive tumor, as assessed by histopathological features, and undergo longer survival, compared with those having a concomitantly occurring thyroiditis (184).

AIM OF THE THESIS

Inflammation is currently regarded as an essential component of malignancies, including differentiated thyroid cancer (DTC). The characteristics of tumor microenvironment can profoundly influence the biological behavior of cancer cells and, ultimately, the patient's outcome. Chemokines and their receptor play a major role in determining the immune phenotype of the cells infiltrating the

thyroid tumor microenvironment. Thyroid cancer microenvironment is composed of a mixture of immune cells and soluble mediators. Among them, the chemokine CXCL8 exerts multiple pro-tumorigenic activities, including a chemotactic action on circulating neutrophils, induction of tumor cells growth, increase in angiogenesis and induction of the epithelial to mesenchymal transition, which promotes cell migration. Clinical studies in patients affected by several types of cancer evidenced that CXCL8 serum levels reflect the tumor burden and are related with the tumor aggressiveness. Solid evidence indicates that CXCL8 targeting can reduce tumor progression. Our group recently demonstrated that different thyroid cancer cell lines, with different mutations, secrete high amounts of CXCL8 and a modulation of CXCL8 levels has been obtained with several compounds, such as metformin and AICAR, in different thyroid cancer cell lines.

Under a more clinical point of view, the controversial relationship between inflammation and thyroid cancer tumorigenesis involves also the debated topic of the association between chronic-autoimmune-thyroiditis (CAT) and differentiated-thyroid-cancer (DTC). DTCs are often diagnosed in the context of CAT and display an inflammatory-immune cells infiltration at histology, but whether the malignant transformation is promoted by the inflammatory response, or the peri-tumoral inflammation is induced by cancer-specific inflammatory molecules is still a matter of debate. Some authors have demonstrated a higher risk of DTC in patients with CAT (especially in surgical series), whereas others (especially cytological series) did not confirm this finding. This discrepancy may be, at least in part, due to the fact that most thyroid cancers are slow growing and asymptomatic malignancies, and their incidence does increase in relation to increasing screening procedures, as is typical of patients with CAT. Moreover, little is known about the impact of thyroid autoimmunity on the prognosis of an already existing DTC. In particular, the role of CAT in determining the cancer microenvironment and chemokine milieu, which are known to deeply influence the prognosis of DTC, is still poorly understood.

This thesis project followed two principal aims:

- Aim 1: to investigate the role of a pro-tumorigenic chemokine (CXCL8) in thyroid cancer microenvironment and to test the modulating properties of two different pharmacologic agents (PLX4720 and phenformin). CXCL8 protumorigenic role was evaluated through both migration assay and cell proliferation tests in primary cultures and lines of thyroid cancerous and normal cells

- Aim 2: to evaluate if CAT is a risk factor for the de novo development of DTC through a longitudinal population study.

MATERIALS AND METHODS

AIM 1

Primary cultures of NHT

Surgical specimens of normal human thyroid were obtained from the contralateral disease-free lobe of patients who underwent thyroidectomy for a solitary non-functioning nodule (n=3). The study was approved by the Institutional Board of ICS-Maugeri. Before surgery, written informed consent to the study was obtained from all patients. All the experiments were performed in accordance with the relevant guidelines and regulations. Surgical specimens were minced and then incubated with collagenase type II (Sigma, Saint Louis, MO, USA) 5 mg/ml, in 5 ml of Coon's F12 medium, for 4h at 37°C as previously described (185). Then, 10 ml of Coon's F12 medium were added, following which, cells were filtered, spun at 1000 x g for 10 min, washed with Coon's F12 medium, spun again, and finally re-suspended in complete medium containing 5% newborn calf serum and a mixture of six hormones including insulin (5μg/ml), hydrocortisone (50 μg/ml), transferrin (5 μg/ml), somatostatin (10 ng/ml), gly-his-lysine (10 ng/ml) and bovine TSH (1 mU/ml).

Thyroid tumor cell lines BCPAP, TPC-1, 8305C and 8505C

Human thyroid cancer cell lines, BCPAP harboring the BRAF V600E mutation and TPC-1 bearing the RET/PTC rearrangement, were a gift of Prof. M. Santoro (Medical School, University "Federico II" of Naples, Naples, Italy). These cell lines had been previously tested and authenticated by DNA analysis. Cancer cells were propagated in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, Saint Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma, Saint Louis, MO, USA), 2mM L-glutamine and 100 U/ml penicillin/streptomycin (Sigma, Saint Louis, MO, USA) as previously described (185) . Human thyroid cancer cell lines, 8305C and 8505C harboring the BRAF V600E mutation were previously tested and authenticated by DNA analysis. 8305C cells were propagated in Mininum Essential Medium (MEM) (Sigma, Saint Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma, Saint Louis, MO, USA), 2mM L-glutamine and 100 U/ml penicillin/streptomycin (Sigma, Saint Louis, MO, USA). 8505C cells were propagated in RPMI

medium (Sigma, Saint Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma, Saint Louis, MO, USA), 2mM L-glutamine and 100 U/ml penicillin/streptomycin (Sigma, Saint Louis, MO, USA). Cells were incubated with the chosen stimuli in serum-free medium.

Evaluation of basal and TNF-α-induced CXCL8 secretion in BCPAP, 8305C, 8505C, TPC-1 thyroid cancer cell lines and in primary cultures of NHT in the presence or absence of increasing concentrations of PLX4720 or phenformin.

For the CXCL8 secretion assays, 3000 cells were seeded into 96-well plates in complete medium. After adherence to the plastic surface, BCPAP, TPC-1, 8305C, 8505C and NHT cells were incubated for 24h in serum-free medium (basal condition) with increasing concentrations of PLX4720 (Sigma Aldrich) or phenformin PLX4720 (Sigma Aldrich). Incubation with PLX4720 was done in the absence (basal secretion) and in the presence (stimulated condition) of TNF-α 10 ng/ml. All experiments were performed in triplicates.

Time course of PLX4720 inhibition of CXCL8 secretion in BCPAP, 8305C, 8505C, TPC-1 thyroid cancer cell lines and in primary cultures of NHT

For the CXCL8 secretion assays, 3000 cells were seeded into 96-well plates in complete medium. After adherence to the plastic surface, BCPAP, 8305C, 8505C and NHT cells were incubated for 24, 48 and 72 hours with or without PLX4720 0, 0.1, 1, 2, 5, 10µM. All experiments were performed in triplicates.

ELISA for CXCL8

CXCL8 was measured in cell supernatants of BCPAP, TPC-1, 8305C, 8505C and NHT cells using commercially available kits (R&D Systems, Minneapolis, MN). The mean minimum detectable concentration of CXCL8 was 3.5 pg/ml. The intra- and inter-assay coefficients of variation were 3.4% and 6.8%, respectively. Samples were assayed in duplicates.

Migration

The cell migration assay was performed with the trans-well migration chamber system (Merck Millipore, Milan, Italy), as previously described (185). Briefly, NHT, TPC-1, BCPAP, 8305C and 8505C were cultured for 24 hours with fresh medium alone or supplemented with 10μM of PLX4720, 100ng/ml of CXCL8 (R&D Systems, Minneapolis) or their combination. After treatment, 20x103 cells/well were seeded in the upper chambers of the 96-well plate with polycarbonate inserts having 0.3 cm2/well membrane area and 8 μm pore size. In each condition the lower chambers were filled with 150 μl of the corresponding medium. Cells were left to migrate for 16 hours at 37°C and 5% CO2. At the end of the incubation, samples were analyzed as previously described (186). Briefly, cell inserts were washed three times with PBS and migrated cells on the underside of the membrane were fixed with 4% paraformaldehyde for 20 minutes. Cell nuclei were then stained with Hoechst 33342 (1:1000) (Life Technologies, Monza, Italy). Finally, the membranes were cut out with a scalpel, and mounted onto glass slides with DACO reagent (Life Technologies, Monza, Italy). Three replicates were evaluated for each condition. Images were acquired using an Olympus BX51 microscope (Olympus, Deutschland GmbH, Hamburg, Germany). The number of migrated cells was counted analyzing 12 random fields of the membranes per condition. Data are expressed as % of mean numbers of migrated cells ± standard deviation (SD).

Cell viability, WST-1 assay

NHT, TPC-1 and 8505C were grown in complete medium until an 80% confluence was reached. Cells were then detached and seeded in 96 well flat plates at a density of $2x10^4$ cell/well. Complete medium was supplemented with increasing concentrations of phenformin (0, 0.001, 0.01, 0.1, 1, 10 mM, Sigma Aldrich), the concentrations of phenformin were chosen based on previous studies (187-189). The incubation times were 7, 14 and 24 hours. At the end of treatment, 20 μl of WST-1 were added to wells; plates were then incubated for 30 minutes at 37°C in a 5% CO_2 atmosphere. WST-1 is a colorimetric reagent, which, after cleavage of a tetrazolium salt, MTS, by mitochondrial dehydrogenases, results in the production of formazan by viable cells

only. Absorbance was then measured at 450 nm by using a multimode plate reader (Victor NIVO Multimode Plate Reader, PerkinElmer). All experiments were performed in triplicates. Values of IC50s (concentrations necessary to reduce the cell viability by 50%) were calculated for the various cell lines from concentration-response curves by linear regression analysis (percent/inhibition against the negative natural logarithm of the molar concentration of phenformin).

Annexin V-FITC/PI assay to detect cell death

The cell surface exposure of phosphatidylserine on the plasma membrane of cells were assessed using Annexin V-FITC Apoptosis Detection Kit (Life-Technologies Apo-Detect Kit). Briefly, cells were harvested on a coverslip sited in a 24-well plate at a density of 10^4 cells per well. After adhesion, cells were treated with phenformin at 0, 1 and 10 mM. After 24 hours of treatment cells were washed with PBS and supernatants were conserved for cyto-spin. Coverslips were incubated with a mix of Annexin V-FITC, PI and Hoechst 33258 (Thermofisher) in the dark for 10 min at room temperature. The same procedure was reserved for cells recovered in the supernatants after centrifugation. Cells were fixed with PFA 4% for 10 minutes. After washing with PBS, coverslips were mounted with Dako and the fluorescence images were obtained with a Leica TCS-SP5 II inverted confocal microscope.

Colony formation assay

Colony formation assay is an *in vitro* cell survival assay that tests the ability of a single cancer cell to undergo ‘‘unlimited’’ division, thus growing into a colony. This is the method of choice to determine the loss of cancer cells replicating capacity after treatment with a given compound (190).

TPC-1 and 8505C cell lines were incubated in the presence or absence of increasing concentrations of phenformin for 24 hours. Cells were detached with trypsin, plated into 24-well plates (2x 10^3 cells/well) and maintained in complete medium for 8 days. Cells were fixed with methanol for 20 minutes and stained with 0.5% crystal violet dye for 5 minutes (191). Colony formation was confirmed under an inverted light microscope Olympus BX51 microscope (Olympus,

Deutschland GmbH, Hamburg, Germany). For quantification, after three washes with deionized water to remove excess stain, the crystal violet dye was released from the cells by incubation with 1% SDS for 2 hours before optical density (OD) 570 nm measurement.

Statistical analysis

Statistical analysis was performed using the SPSS software (SPSS, Inc., Evanston, IL). Mean group values were compared by using one-way ANOVA for normally distributed variables. Post hoc analysis was performed according to the Bonferroni's correction for multiple comparisons. Values are reported as mean ± SD unless otherwise noted. A p value < 0.05 was considered statistically significant

AIM 2

Patients and Methods:

The outpatients' Data Base of the Unit of Endocrinology of ICS Maugeri (Pavia, Italy) was searched for patients who received a diagnosis of CAT between 1997 and 2014. Pavia, located in Northern Italy, is an area of mild iodine deficiency. Inclusion criteria were: 1) a diagnosis of CAT, as assessed by positive tests for circulating thyroglobulin (TgAb) and/or thyroid peroxidase (TPOAb) antibodies, and/or a hypoechoic pattern of the gland at US; 2) the availability of a thyroid US at diagnosis; 3) a benign cytology in patients initially presenting an associated thyroid nodule (NOD); 4) a minimal follow-up period of 5 years, with an yearly thyroid US scan performed by the same operator.

The final study cohort included 510 patients (476 females and 34 males). In details, 297 (58.2%) patients showed positive tests for both TgAb and TPOAb; 134 (26.3%) patients had only TPO Ab; 44 (8.6%) had only Tg Ab; 35 (6.9%) patients had negative tests for both Ab and based upon a diffuse hypoechoic pattern of the thyroid at ultrasound were diagnosed with serum negative autoimmune thyroiditis (133,192) . Their median (range) age was 49 (19-82 years).

Patients were further subdivided in 2 groups according to the presence or absence of co-existent nodule/s at diagnosis. Group 1 (CAT+, NOD+) and group 2 (CAT+, NOD-) included 115 and 395 patients, respectively.

The median (range) follow-up period was 10 (5-17) years. The development of new thyroid nodule/s and/or significant modifications in pre-existent nodules were registered throughout the follow-up.

All patients signed an informed consent concerning the future use of their clinical-pathological data for research purposes. This study was approved by the Ethical Committee of ICS Maugeri, Pavia (Protocol N. 2403 CE).

Thyroid ultrasound

Thyroid US evaluation was performed using a real-time US device equipped with a linear transducer operating at 7.5 MHz. All thyroid US scans were performed by the same experienced operator. Thyroid nodules were examined for the following US features: nature (I.e., solid, cystic, or mixed); echogenicity (i.e., hyperechoic, isoechoic, or hypoechoic compared with normal thyroid parenchyma and with neck muscles); size, homogeneity (i.e., homogeneous or inhomogeneous); microcalcifications (hyperechoic spots of at least 2 mm without acoustic shadowing); regular or irregular margins; halo sign (i.e., presence or absence of an hypoechoic rim); and pattern of color-flow Doppler. The above mentioned characteristics were summarized according to the EU-TIRADS classification system(193). Thyroid volume was estimated by the elliptical shape volume formula (length x width x depth x 0.479). Lateral neck lymph nodes were routinely examined.

Fine needle-aspiration cytology (FNAC)

FNAC was performed in all newly developing nodules when they were larger than 1 cm in maximum diameter or, irrespective of their size, when they showed suspicious features at US. FNAC was performed under US guidance by a skilled endocrinologist using a 23-gauge needle attached to a 2.5-mL syringe. Cytological samples were classified into 6 classes: THY1 (nondiagnostic), THY2

(benign), THY3 (Atypia of undetermined significance or follicular lesion of undetermined significance), THY4 (Follicular neoplasm or suspicious for a follicular neoplasm), THY5 (suspicious for malignancy), and THY6 (malignant), according to the Bethesda classification (31). None of the lymph-node was found to require FNAC.

Statistical Analysis

Statistical analysis was performed using the SPSS Software (SPSS, Inc.). Between-groups comparisons were performed using the Student's t-test for unpaired data and the Mann–Whitney U-test according to a normal or a non-parametric distribution. Within-group comparisons were performed using the Student's t-test for paired data and the Wilcoxon's test according to a normal or a non-parametric distribution. Frequencies among groups were compared using the $\chi 2$-test with Fisher's correction when appropriate. To test the effects of different variables independent of a covariate, multivariate logistic regression analysis was used and partial correlation coefficients were computed. The multivariate model was constructed by entering the occurrence of a new nodule as a dependent variable, while age, gender, thyroid volume at baseline and the Group (CAT+ NOD+ and CAT+ NOD-) were entered as covariates.

Kaplan-Meier estimates were used to generate an overall survival curve for new nodules occurrence and differences among CAT+ NOD+ and CAT+ NOD- groups were assessed by log-rank test. A p value of <0.05 was considered statistically significant. Results are expressed as mean ±sd, unless otherwise stated.

RESULTS

AIM 1

Effects of treatment with PLX4720 in terms of inhibition of CXCL8 secretion in thyroid cancer cell lines and NHT.

CXCL8 concentrations were assayed in the supernatants of, BCPAP, 8305C and 8505C in basal and TNF-α-stimulated condition. As previously reported, TNF-α elicited a significant increase in the concentrations of CXCL8 in the supernatants of BCPAP (80,145) but also in 8305C and 8505C cells (data not shown). The treatment with PLX4720 significantly and in a dose-dependent manner, inhibited the secretion of CXCL8 in: BCPAP **(Figure 1, Panel A)**, in 8305C **(Figure 1, Panel B)** and in 8505C **(Figure 1, Panel C).** *Post Hoc* analysis performed by Bonferroni demonstrated some differences in the strength of inhibition induced by PLX4720 in different cell types. Indeed, significant inhibition of the basal secretion of CXCL8 started from a 2μM concentration of PLX4720 in BCPAP **(Figure 1, Panel A)** and from a 0.1 μM concentration of PLX4720 in 8305C **(Figure 1, Panel B)** and 8505C **(Figure 1, Panel C).**

PLX4720 also produced a significant and dose-dependent inhibition of the TNFα-induced CXCL8 secretion in BCPAP cells **(Figure 1, Panel F),** in 8305C cells **(Figure 1, Panel G)** and in 8505C cells **(Figure 1, Panel H).** Also in this case, some differences among cell types were observed. Indeed, *Post Hoc* analysis by Bonferroni showed that the CXCL8 inhibiting effect of PLX4720 was significant starting from 1 μM in BCPAP **(Figure 1, panel F)** and in 8305C **(Figure 1, Panel G)** while in 8505C, a significant inhibition of the TNF-α-stimulated CXCL8 secretion started from a 0.1 μM concentration of PLX4720 **(Figure 1, Panel H).**

At difference with the above findings, PLX4720 did not produce any effect in terms of CXCL8 inhibition on TPC-1 cell lines, both in basal and in TNF-α-stimulated conditions **(Figure 1, Panel D-I).** However, treatment with PLX4720 inhibited both the basal and the TNF-α-stimulated CXCL8 secretion in NHT (Figure 1 E-L). *Post Hoc* analysis by Bonferroni evidenced a significant inhibition of the basal and the TNF-α-stimulated CXCL8 secretion only at the highest (10μM) PLX4720 concentration **(Figure 1, Panel E-L).**

Time course of PLX4720 inhibition of CXCL8 secretion

To assess whether the CXCL8-inhibiting effect of PLX4720 could vary over time, a time course of CXCL8 secretion following treatment with PLX4720 was performed. For this set of experiments those cells in which an inhibitory effect on CXCL8 secretion was exerted by PLX4720 (NHT, BCPAP, 8505C and 8305C) were treated with PLX4720 (0, 0.1, 1, 2, 5, 10μM) for 24, 48 and 72 hours. A first finding was that, throughout the time course the basal levels of CXCL8 progressively increased in all cell types (although at a different magnitude) **(Figure 2, Panel A-B-C-D).** The percentages of inhibition of the CXCL8 secretion were compared for each concentration of PLX4720 at 24-h, 48-h and 72-h in each cell type. Separated ANOVAs for each cell type were performed to assess possible differences in percentages of inhibition of CXCL8 secretion by PLX4720 at each concentration throughout the time course. As shown in (**Figure 3, Panel A-B-C-D)**, no significant change in the inhibitory power of a given concentration of PLX4720 as assessed at 24h, 48h and 72 h could be found in any cell type. These findings would suggest that the CXCL8-inhibitory effect of PLX4720 is maintained (at a similar strength of inhibition) up to 72 hours.

Migration Assays

To assess whether the PLX4720-mediated inhibition of CXCL8 secretion would produce any biological consequence, cell migration assays were performed.

The transwell migration assay showed that rh-CXCL8 induced migration in all cell types. In details, the following results were found: 150 ± 20 % migrated cells in BCPAP, 150 ± 0.1 % migrated cells in 8305C, 120 ± 30 % migrated cells in 8505C, 130±20 % migrated cells in NHT, 140 ±10 % migrated cells in TPC-1, **Figure 4 Panel A-B-C-D-E**. The incubation with PLX4720 inhibited basal cell migration of BCPAP, 8305C, 8505C and NHT, **Figure 4 Panel A-B-C-D**. On the other hand, PLX4720 did not inhibit the basal migration of TPC-1 **Figure 4 Panel E**. Finally, co-treatment with PLX4720 and rh-CXCL8 had an inhibitory effect on the CXCL8-induced cell migration of BCPAP, 8305C, 8505C and NHT but not in TPC-1 cells **Figure 4 (A-B-C-D-E).**

Effect of phenformin on NHT, TPC-1 and 8505C thyroid cells viability

To assess changes in thyroid cells viability, a time-course incubation experiment was performed. Cells were incubated for 7, 14 and 24 hours in the presence of increasing concentrations of phenformin. As shown in **Figure 5 (Panel A-B-C),** treatment with phenformin reduced TPC-1 cell viability in a time- and dose-dependent manner. Incubation with 10 mM phenformin reduced cell viability after 7 hours (**Figure 5 Panel A)**. A more pronounced effect on TPC-1 cell viability was observed after a longer exposure time even at lower concentrations of phenformin. Significant reduction of TPC1 cell viability was observed starting from 0.1 mM concentration **(Figure 5 Panel B)** after 14 hours and starting from 0.001 mM after 24 hours (**Figure 5 Panel C**). Similarly, in 8505C, phenformin reduced cell viability starting from a 7-hour incubation time but only at the maximal concentration of 10 mM **(Figure 5 panel D).** Significant reduction of 8505C cell viability was observed starting from a 0.1 mM concentration after 14 hours **(Figure 5 Panel E)** and after 24 hour of treatment **(Figure 5 Panel F).** Unlike thyroid cancer cells, phenformin did not reduce viability in NHT cells after a 7 hour incubation time at any of the used concentrations **(Figure 5 Panel G)**. A reduction of NHT cells viability was observed only at the maximal concentration of phenformin (10 mM) after 14 and 24 hours **(Figure 5 Panel H-I)** of incubation.

The results of these cell viability experiments were confirmed by staining cells with Annexin V/PI, as shown in **Figure 6**. In details, both thyroid cancer cell lines, TPC-1 and 8505C, treated with 1 mM phenformin show Annexin V positive staining, which marks the exposure of phosphadidylserine at the outer leaflet with intact membrane integrity, but no PI fluorescence, suggesting an early stage of apoptotic events. NHT cells treated with the same concentrations of phenformin did not show Annexin V/PI staining, indicating that these cells were viable. The cells exposed to 10 mM phenformin, by contrast, are clearly positive for both Annexin V and PI fluorescence, which is indicative of loss of membrane integrity typical of a later apoptosis in all cell types.

The IC50 for treatment with phenformin at 24 hours was 7.4 mM for 8505C and 4.08 mM for TPC-1 cells, respectively. An IC50 could not be calculated for NHT cells as none of the tested concentrations elicited a 50% of reduction of cell viability.

Basal secretion of the pro-tumorigenic chemokine CXCL8 after 7, 14 and 24 hours in NHT, TPC1 and 8505C cells.

The levels of CXCL8 were measured in the supernatants from NHT, TPC-1, and 8505C cells in basal condition (**Figure 7**). The concentration of CXCL8 in the cell supernatant increased during the experiment time-course in NHT, in 8505C and in TPC-1 cells, as shown in **Figure 7**. The absolute amounts of secreted CXCL8 greatly differed among normal and malignant cells. TPC-1 cells secreted the greatest amounts of CXCL8 while NHT produced the smallest ones. As shown in **Figure 7,** after a 7-hour incubation period CXCL8 levels were higher in TPC-1 supernatants as compared with the NHT and 8505C ones. After 14 hours of incubation, TPC-1 cell again secreted the greatest amounts of CXCL8, followed by 8505C cells, which secreted higher levels as compared with NHT cells. A similar secretion gradient was observed after 24 hours: TPC-1 > 8505C > NHT cells.

Inhibiting effect of phenformin on CXCL8 secretion

The further step was to evaluate whether phenformin could exert any effect at concentrations lower than those required to induce cell death. To this aim, concentrations of phenformin not affecting cell viability at each time were tested for their ability to modulate the secretion of CXCL8 in TPC-1, 8505C and NHT cells. CXCL8 concentrations were assayed in the supernatants of, NHT, TPC-1 and 8505C at different times and phenformin concentrations. Phenformin inhibited CXCL8 secretion after 7 and 24 hours in NHT cells **(Figure 8 Panel A-B-C)**. No inhibition of CXCL8 secretion was observed in TPC-1 and 8505C cells after 7, 14 and 24 hours of treatment with phenformin at concentrations not affecting cell viability (**Figure 8 Panel D-H).**

Effects of phenformin on the ability of TPC-1 and 8505C to form colonies

The colony formation assay detects cells that have retained the capacity to produce a large number of progeny after pharmacological treatment. As shown in **Figure 9** and **Figure 10,** treatment with phenformin at increasing concentrations reduced in a dose dependent manner the ability of both TPC-1 and 8505C to form colonies. Indeed, the percentage of colony formation was reduced by phenformin starting from the concentration of 0.1 mM in both cell types (**Figure 9-10**).

AIM 2

Table 1 shows demographic data and thyroid US features in CAT+ NOD+ and in CAT+ NOD- groups. Patients in the 2 subgroups had a similar gender ratio, but those in the CAT+ NOD + group were older and had a larger thyroid gland at diagnosis. The two groups were characterized by similar frequencies of patients displaying both Tg Ab and TPO Ab, TPO Ab alone, Tg Ab alone and negative tests for Tg Ab and TPO Ab in the presence of a diffuse hypoechoic pattern of the thyroid parenchyma at US. Compared to the CAT+ NOD+ group, the CAT+ NOD- one comprised a higher percentage of patients who were on LT4 replacement therapy when entered the study or who were started on LT4 replacement therapy just at the beginning of the follow-up. In the CAT+ NOD- group 143/395 (36.2%) patients were receiving LT4 when entered the study, the correspondent figure in the CAT+ NOD+ group was 29/115 (25.2%); $p<0.05$. The median serum level of TSH at study entry was higher in the CAT+ NOD- as compared to CAT+ NOD+ (4.38 vs 2.14 μU/ml; $p<0.001$) group. At the last evaluation the difference was less evident although still significant (2.4 vs 1.76 μU/ml; $p<0.05$). Similarly, 154/252 (61.1%) patients in the CAT+ NOD- group and 31/86 (36.0%) in the CAT+ NOD+ one ($p<0.001$) were started on LT4 when recruited. The overall median and range follow-up time was 10 yrs. (5-17) in the whole cohort, without significant differences between the two subgroups [11 (5-16) yrs. for CAT+ NOD+ vs 10 (5-17) yrs. for CAT+ NOD-; $p=0.083$].

Throughout the follow-up period, the US appearance of new thyroid nodules was detected in 75 out of 510 (14.7%) patients of the whole cohort. When patients' subgroups were analyzed

separately, findings differed in that 34 out of 115 (29.5%) patients in the CAT+ NOD+ subgroup developed new nodules as compared with a significantly lower frequency in the CAT+ NOD- subgroup: 41 out of 395 (10.3%) patients (p<0.001) **(Figure 11)**. The median and range time for new nodules appearance was 4 (1-13) yrs. As shown in **Figure 12**, life time survival analysis for new nodule development confirmed a significant difference between the two subgroups, with a higher risk for new nodule development in the CAT+ NOD+ subgroup (Kaplan-Meier Log Rank 24.441, p<0.001).

Twenty-seven out of 75 (36%) patients experiencing the appearance of a new nodule met the US criteria for performing a FNAC. In 23 of them (85% of cases) FNAC was performed due to the detection of a larger than >1 cm in maximum diameter nodule, whereas in 4 patients (15%) the indication for FNAC derived from suspicious US features. No difference in the rate of nodules requiring FNAC was observed in the two subgroups of patients (14 out of 34 patients in the CAT+ NOD+ and 13 out of 41 patients in the CAT+ NOD-; p = 0.472). A benign cytology was observed in all the 27 patients who underwent FNAC **(Table 2).** The ultrasound characteristics at the end of the follow-up of both nodules requiring or not requiring FNAC are shown in Table 2. We also compared the median follow-up time calculated from the first ultrasound detection of a new nodule and the end of the follow-up in the two subgroups of nodules [6 (0-11) yrs. versus 3.5 (0-11) yrs, respectively; p=0.051]. Although statistical significance was not reached, nodules requiring FNAC showed a trend for a longer follow-up time. **Table 2** summarizes the ultrasound features at the end of the follow-up of nodules requiring or not requiring FNAC.

The different rate of new nodule appearance between the two subgroups prompted further investigation. A multiple binary logistic regression model was constructed entering the appearance of new nodules (yes/no) as dependent variable and age, sex, thyroid volume at diagnosis, subgroup of patients (CAT+ NOD+/CAT+ NOD-), and number of nodules at first US as covariates. As shown in **Table 3**, multiple regression analysis confirmed that patients in the CAT+ NOD+ subgroup carried

a significantly higher risk for developing new thyroid nodules throughout the follow-up, independently of age and sex. Thyroid volume at diagnosis also independently predicted the risk of developing new nodules.

DISCUSSION

The first aim of this thesis was to evaluate the role of a pro-tumorigenic chemokine, CXCL8, in thyroid cancer microenvironment and to test the modulating properties of two different pharmacologic agents (PLX4720 and phenformin).

First, our *in vitro* data confirmed the pivotal role of CXCL8 in thyroid cancer behavior. We demonstrated that CXCL8 is actively secreted by thyroid cells, as measured in cellular supernatants, but in much higher quantities in cancerous cells when compared with normal cells. Moreover, the specific pro-tumorigenic mutation harbored by the different cell lines directly influences the entity of CXCL8 secretion: the secreted quantity was greatest in the RET/PTC mutated cell line (TPC-1), followed by BRAF-mutated cell lines (BCPAP, 8505C and 8305C). These data confirm that the underlying genetic mutation induces different patterns of CXCL8 secretion (76,80,115). NHT cells were able to secrete CXCL8, even if at much lower concentrations.CXCL8 secretion was also up-regulated after TNF-α treatment, further confirming previous studies form our group (80,116).

As a second point, our *in vitro* data further confirmed the important pro-tumorigenic effect exerted by CXCL8 on thyroid cancer cells. In detail, after treatment with recombinant CXCL8 an increase in cell migration ability was observed through trans-well migration assay in all cell types. This phenomenon suggests that CXCL8 can increase the migration ability of cancerous cells, and thus their capability of causing dissemination and long-distance metastasis (194).

As a third point, the treatment of the different cell tipes with two emerging compounds with anti-cancer potential properties (PLX 4720 and Phenformin) was able to inhibit, with different modalities, CXCL8 secretion and thus reduce the pro-tumorigenic effect of this chemokine.(195)

PLX4720, a selective BRAF-inhibitor, was able to inhibit both the basal and the TNFα-stimulated secretion of CXCL8 in BRAFV600E mutated thyroid cancer cell lines (BCPAP, 8305C and 8505C) and also in NHT, even if only at the maximal concentration. On the other hand, PLX4720 did not produce any significant inhibition of CXCL8 inhibition in TPC-1 cells harboring RET-PTC rearrangement. The inhibition observed in NHT cells is in line with previous observations regarding a certain degree of anti-cancer effect observed by treatment with PLX4720 at high concentrations in BRAF-wild type cells (196). The inhibitory effect of PLX4720 treatment on CXCL8 secretion was maintained after a 72 hours-long exposure, suggesting the lack of a rebound effect in these conditions. (197). Moreover, the results of migration assays experiments indicated that PLX4720 was able to reduce the migration of those types of thyroid cells in which the treatment by PLX4720 also produced an inhibition of the CXCL8 secretion (BCPAP, 8305C, 8505C, NHT) but not in those cells in which PLX4720 did not inhibit CXCL8 secretion (TPC-1). These data are in line with previous evidence showing several anti-cancer effects of PLX4720 in thyroid cancer cells, including a reduction of proliferation, migration and invasion ability(198,199), and suggest that these effects, at least in part, could be due to an inhibition of CXCL8 secretion.(54)

We then examined the possible anti-cancer effects of another compound, phenformin, a biguanide. In this case, the main anti-cancer effect of the compound was a time and dose-dependent cytotoxic effect in thyroid cancer cell lines 8505C and TPC-1. On the contrary, only a slight cytotoxic effect, at high doses and after long-term treatment, was witnessed in NHT cells. These results suggest that normal and thyroid cancer cells profoundly differ in their response to phenformin in terms of cell death induction, and that normal thyroid cells could be preserved during an hypothetical anti-cancer treatment with phenformin. (200)

A second finding of our study was that phenformin, at non-cytotoxic concentrations, could inhibit the secretion of CXCL8 only in normal thyroid cells. Thus, in addition to a direct anti-tumor effect (induction of cell death), phenformin also exerts indirect anti-tumor effects through a modification of

the chemokine milieu within the tumor microenvironment. This finding is particularly relevant because NHT are the most represented cells in thyroid cancer microenvironment.

Lastly, phenformin treatment reduced the ability of thyroid cancer cells to form colonies, suggesting that the potential benefits of this molecule as an anti-cancer drug could include the eradication of cancer cells with unlimited proliferating ability required for the prevention of recurrences (190).

For both compounds, our study was not directly aimed at understanding the physiopathogenetic mechanisms of the anti-cancer activity. In the case of PLX4720, it is highly probable that its mechanism of action in directly linked with BRAF-inhibition in BRAF-mutated cells, although the presence of a certain CXCL8 lowering effect of the drug also in NHT cells suggests that other mechanisms could be involved. In the case of phenformin, its cytotoxic effect could be directly proportional to the proliferative rate of different cell types. Indeed, it was previously reported that higher proliferation rate characterizes TPC-1 cells as compared to 8505C (201) and proliferation rate of NHT primary cells is clearly lower when compared with that of thyroid cancer cell lines (202). The fact that the pattern of CXCL8 inhibition varied greatly according to different cancer cell types and the compound employed is not surprising, since the pathway of CXCL8 secretion greatly differs according to the underlying genetic alterations (80). Therefore, since the mechanisms of CXCL8 secretion differ among cell types, it appears not surprising that also pharmacological inhibiting strategies would be different.

Independently form the possible mechanism, the fact that both tested agents have a direct impact on CXCL8 secretion in cancer microenvironment *in vitro* has important implications on their anti-cancer potential also in clinical practice.

Several agents were previously reported to have an inhibiting effect on the secretion of CXCL8 in thyroid cancer microenvironment. These include AICAR, Interferons and also the biguanide metformin (116,127-129,145,203). CXCL8 is a chemokine with extensively described pro-tumorigenic effects which include influencing of tumor cell growth, angiogenesis, invasiveness and

EMT(204-207) . Furthermore targeting/lowering of CXCL8 levels is known to produce beneficial effects in thyroid cancer. Interestingly, a previous *in vivo* study showed that patients with melanoma harboring the BRAFV600E mutation experience a significant decrease in the circulating levels of CXCL8 while treated with BRAF-inhibitors (208). Our in vitro data strongly support the potential anti-cancer effect of these two emerging drugs (PLX4720 and phenformin) in thyroid cancer, for which clinical studies are warrented.

The second aim of this thesis was to evaluate the role of a classic example of chronic inflammation, namely chronic autoimmune thyroiditis (CAT), as a risk factor for the onset of differentiated thyroid cancer (DTC). For this aim, we designed a large cohort study including patients with CAT followed by US for a median time of 10.0 years. Our results showed that the appearance of new thyroid nodules is not a rare event in patients with CAT, and that new nodules appeared at significantly higher rates in CAT patients with thyroid nodules at diagnosis compared with those having a nodule-free CAT. Nevertheless, all newly appeared thyroid nodules were benign at FNAC analysis. Multivariate analysis identified thyroid volume and presence or absence of nodule/s at diagnosis as significantly related covariates for new-nodule development.

The main result of this clinical study was that, during a 10-year follow-up observation, CAT did not represent a risk factor for the development of clinically overt DTC. This finding argues against the hypothesis that CAT is pathogenically involved in the development of DTC. Previous studies highlighted the role of higher serum levels of TSH as a risk factor for DTC development(209) being nodular autonomy a protective factor (210,211). Furthermore, it was reported that Tg Ab but not TPO Ab would be associated with increased frequency of DTC (156,212). However, since in the present study, no case of DTC was observed throughout the follow-up, neither the role of serum TSH levels nor that of specific thyroid circulating antibody could be addressed. It should be noted that the limited number of nodules eligible for FNAB in our series could represent a limitation of this study.(213)

The results of the present study are in agreement with previous studies, that failed to show an association between the presence of CAT and the onset of DTC during a long term follow-up (169,174). Based on our and previous data, it appears that, when evaluating the risk for DTC in patients with CAT, a great discrepancy exists between cross-sectional, mainly surgical, studies, which consistently report an association, and longitudinal investigations, which fail to support this relationship. Thus, it is conceivable to believe that routine thyroid ultrasound examination of CAT patients is responsible for the high prevalence of DTC in cross sectional studies.

Taken together the above reported data support the hypothesis that patients with CAT are not characterized by an increased occurrence of clinically overt DTC, even in the long-term. Consistent with this statement is the study by Dvorkin et al (162) who reported that, at diagnosis, CAT-associated DTCs are small in size and have a low rate of nodal involvement. These peculiarities suggest an earlier diagnosis and indirectly support the concept that CAT patients represent a more ultrasound screened population (162).

These clinical data support the concept that the relationship between inflammation and cancerogenesis is more complex than initially imagined, and that different kinds of inflammation would have opposing effects in cancer microenvironment. Our hypothesis is that in the case of DTC, the presence of CAT and thus autoimmunity-driven inflammation, does not confer and increase in the pro-tumorigenic potential within the cancer microenvironment, but instead could induce a tumor-controlling environment. This hypothesis is supported by recent pre-clinical studies (184) as well as clinical prospective studies (177,214,215), showing that patients with pre-existing CAT have milder forms of DTC and experience better long term-outcomes. One of the possible explanations of this phenomenon resides in the different chemokine milieu that characterizes CAT patients, when compared with those without CAT. The high concentrations of IFN-inducible chemokines like CXCL10 would induce an immune infiltrate characterized by high numbers of antigen-presenting cells like dendritic cells and effector cells like T-effector lymphocites that favor the control of cancer

cell growth by the host immune system(61,74,75). Moreover, CXCL10 has anti-angiogenetic properties that further contribute to the reduction of metastatic spread of cancer (101,216).

The role of different types of inflammation (tumor-driven versus autoimmune) in thyroid cancer microenvironment in the focus of an ongoing project that will have the aim of evaluating the role of CAT in determining the chemokine balance of DTC microenvironment and, consequently, its clinical behavior.

Published studies:

Coperchini F, Croce L, Denegri M, Awwad O, Ngnitejeu ST, Muzza M, Capelli V, Latrofa F, Persani L, Chiovato L, et al. 2019c The BRAF-inhibitor PLX4720 inhibits CXCL8 secretion in BRAFV600E mutated and normal thyroid cells: a further anti-cancer effect of BRAF-inhibitors. Sci Rep 9 4390.

Coperchini F, Croce L, Denegri M, Awwad O, Ngnitejeu ST, Magri F, Chiovato L & Rotondi M 2019b The anti-cancer effects of phenformin in thyroid cancer cell lines and in normal thyrocytes. Oncotarget 10 6432-6443.

Croce L, Coperchini F, Magri F, Chiovato L & Rotondi M 2019 The multifaceted anti-cancer effects of BRAF-inhibitors. Oncotarget 10 6623-6640.

Rotondi M, Groppelli G, Croce L, Latrofa F, Ancona G, Coperchini F, Pasquali D, Cappelli C, Fugazza A, Guazzoni V, et al. 2020 Patients with chronic autoimmune thyroiditis are not at higher risk for developing clinically overt thyroid cancer: a 10-year follow-up study. *Eur J Endocrinol* 183 317-323.

FIGURES

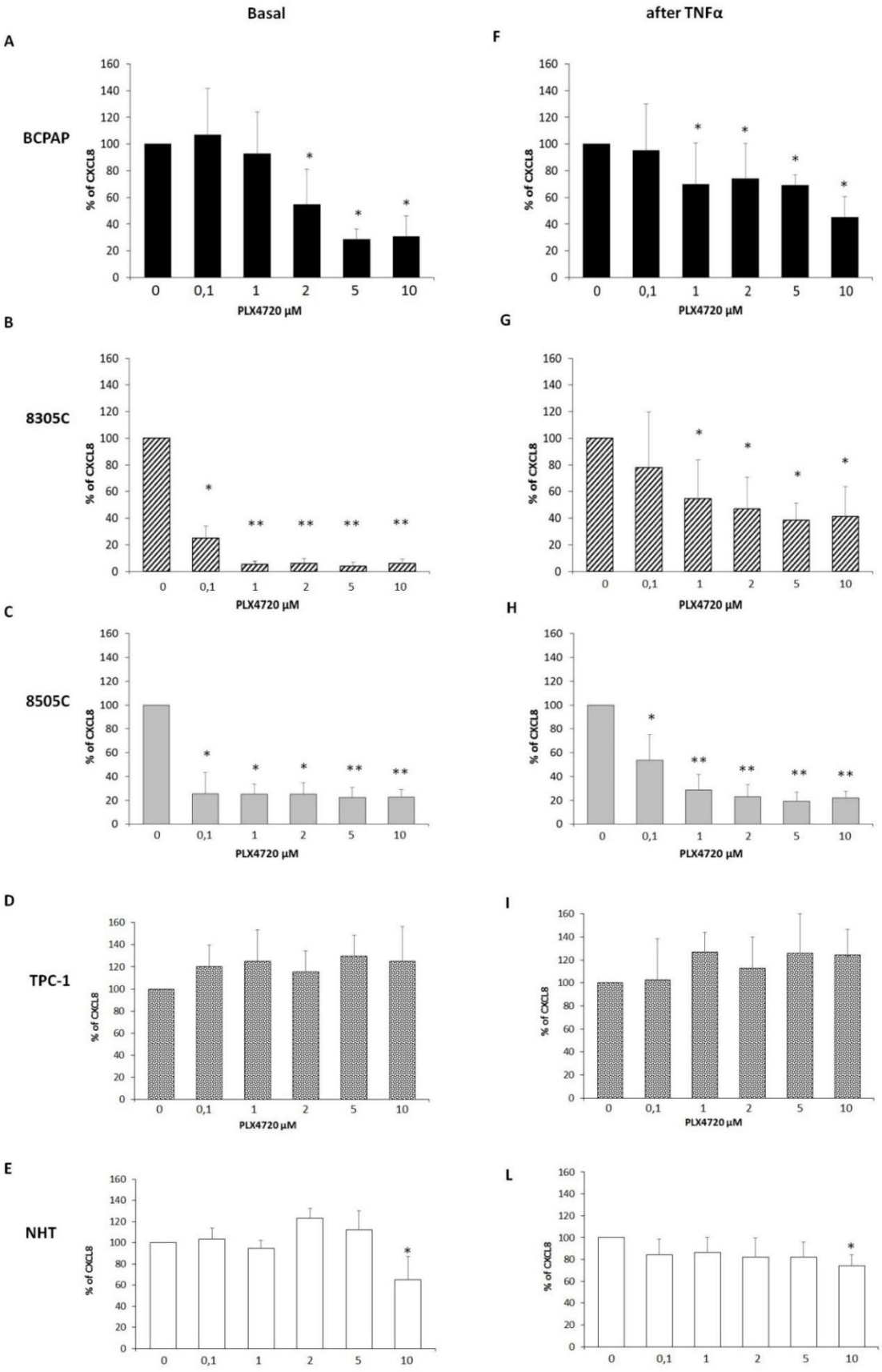

Figure 1

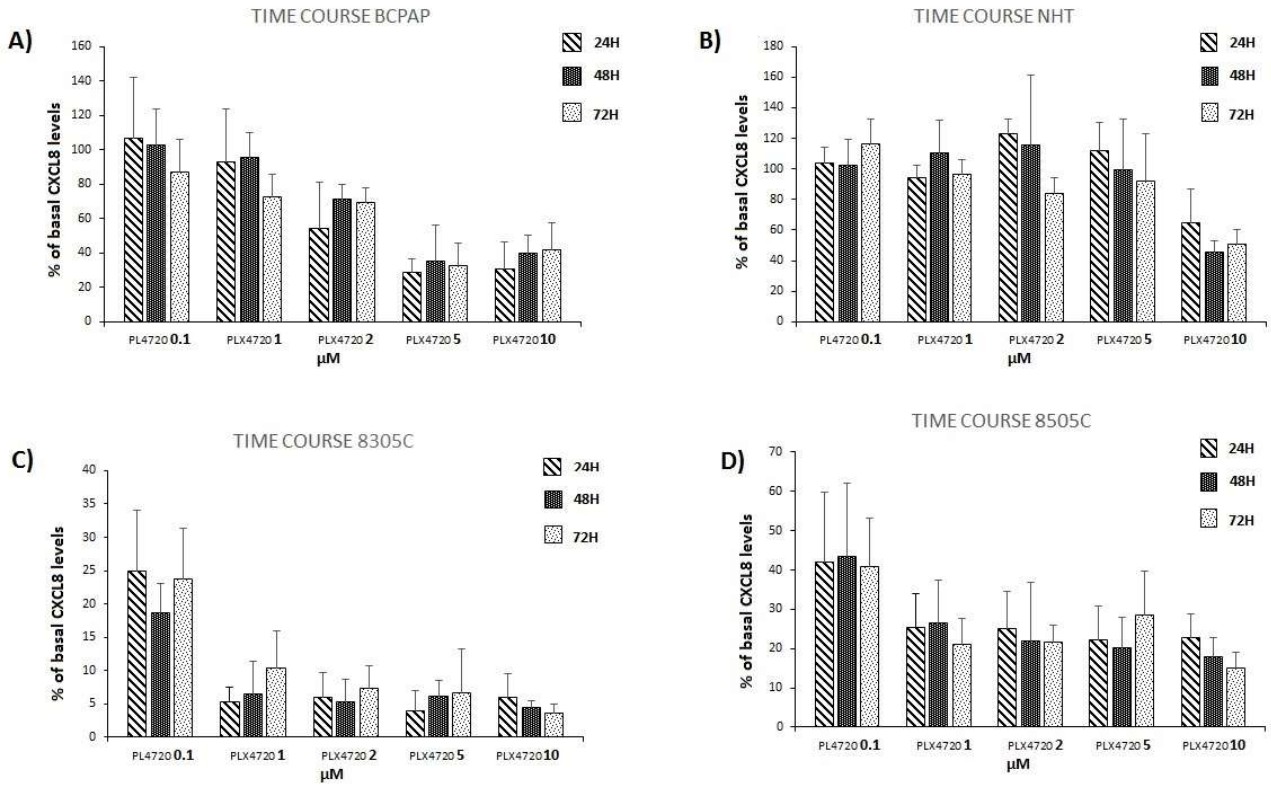
A)
TIME COURSE BCPAP
% of basal CXCL8 levels
24H
48H
72H
PL4720 0.1
PLX4720 1
PLX4720 2
PLX4720 5
PLX4720 10
µM
B)
TIME COURSE NHT
% of basal CXCL8 levels
24H
48H
72H
PL4720 0.1
PLX4720 1
PLX4720 2
PLX4720 5
PLX4720 10
µM
C)
TIME COURSE 8305C
% of basal CXCL8 levels
24H
48H
72H
PL4720 0.1
PLX4720 1
PLX4720 2
PLX4720 5
PLX4720 10
µM
D)
TIME COURSE 8505C
% of basal CXCL8 levels
24H
48H
72H
PL4720 0.1
PLX4720 1
PLX4720 2
PLX4720 5
PLX4720 10
µM

Figure 2

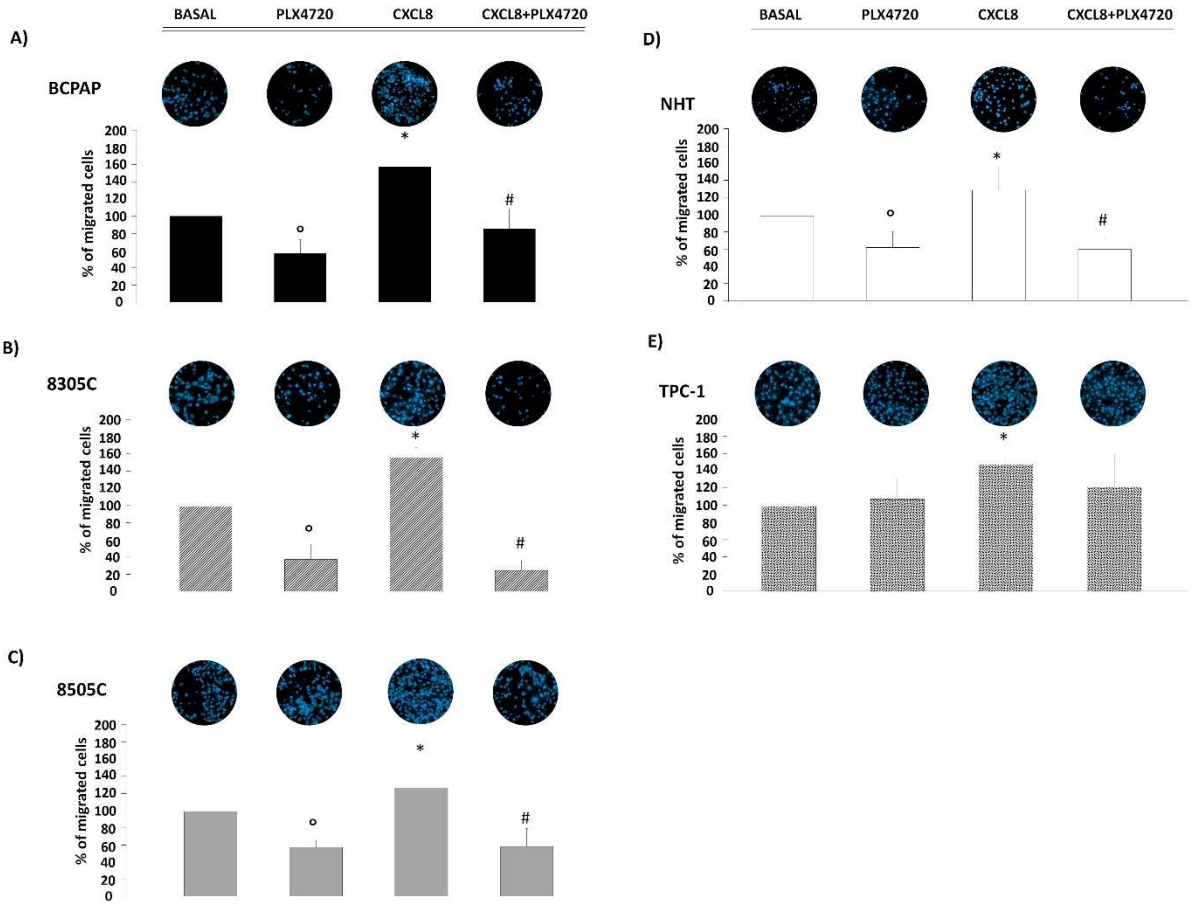
A)
BASAL
PLX4720
CXCL8
CXCL8+PLX4720
BCPAP
% of migrated cells
200
180
160
140
120
100
80
60
40
20
0
*
°
#
B)
8305C
C)
8505C
D)
NHT
E)
TPC-1

Figure 3

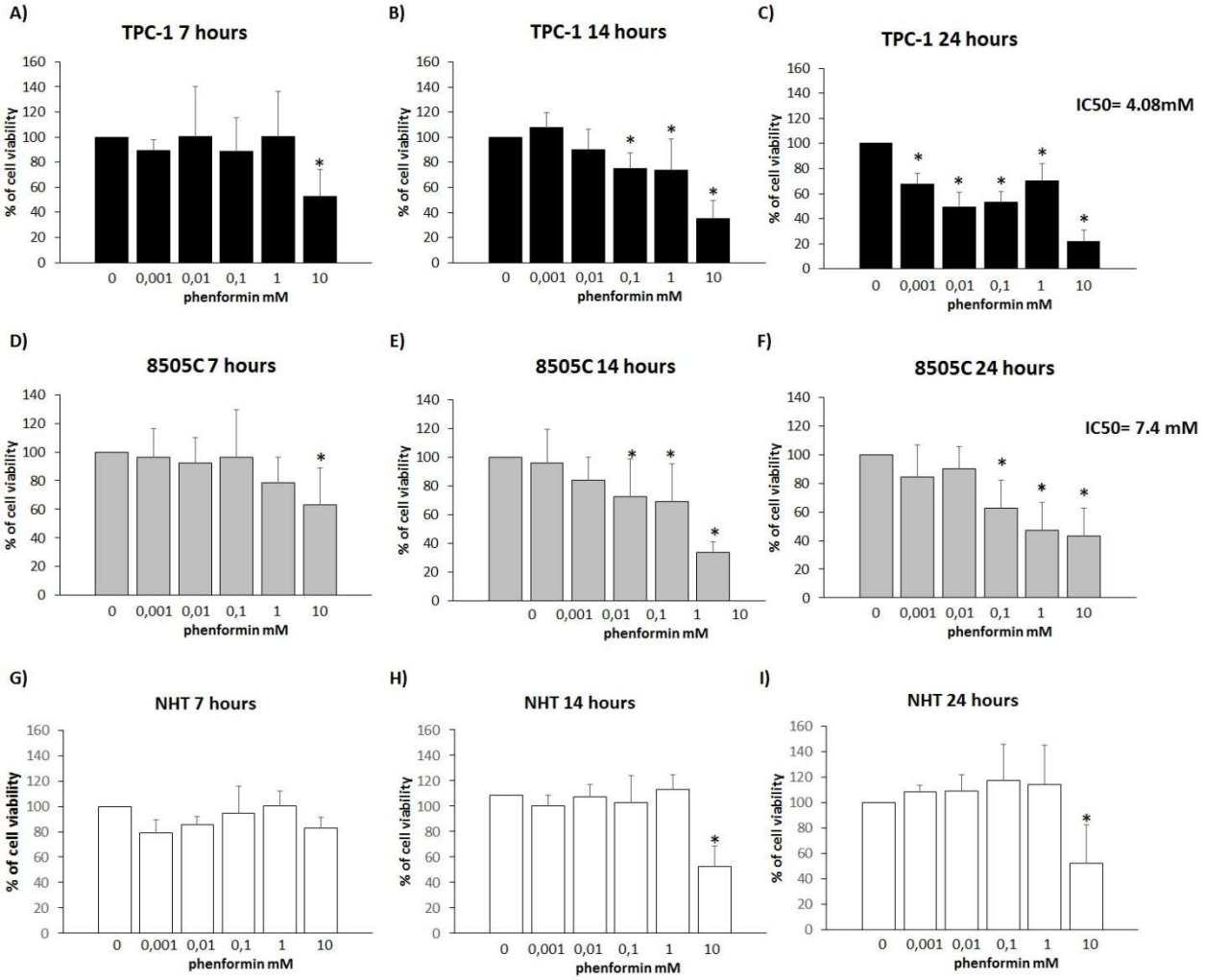
A)
TPC-1 7 hours
B)
TPC-1 14 hours
C)
TPC-1 24 hours
IC50= 4.08mM
D)
8505C 7 hours
E)
8505C 14 hours
F)
8505C 24 hours
IC50= 7.4 mM
G)
NHT 7 hours
H)
NHT 14 hours
I)
NHT 24 hours
% of cell viability
phenformin mM
0
0,001
0,01
0,1
1
10

Figure 4

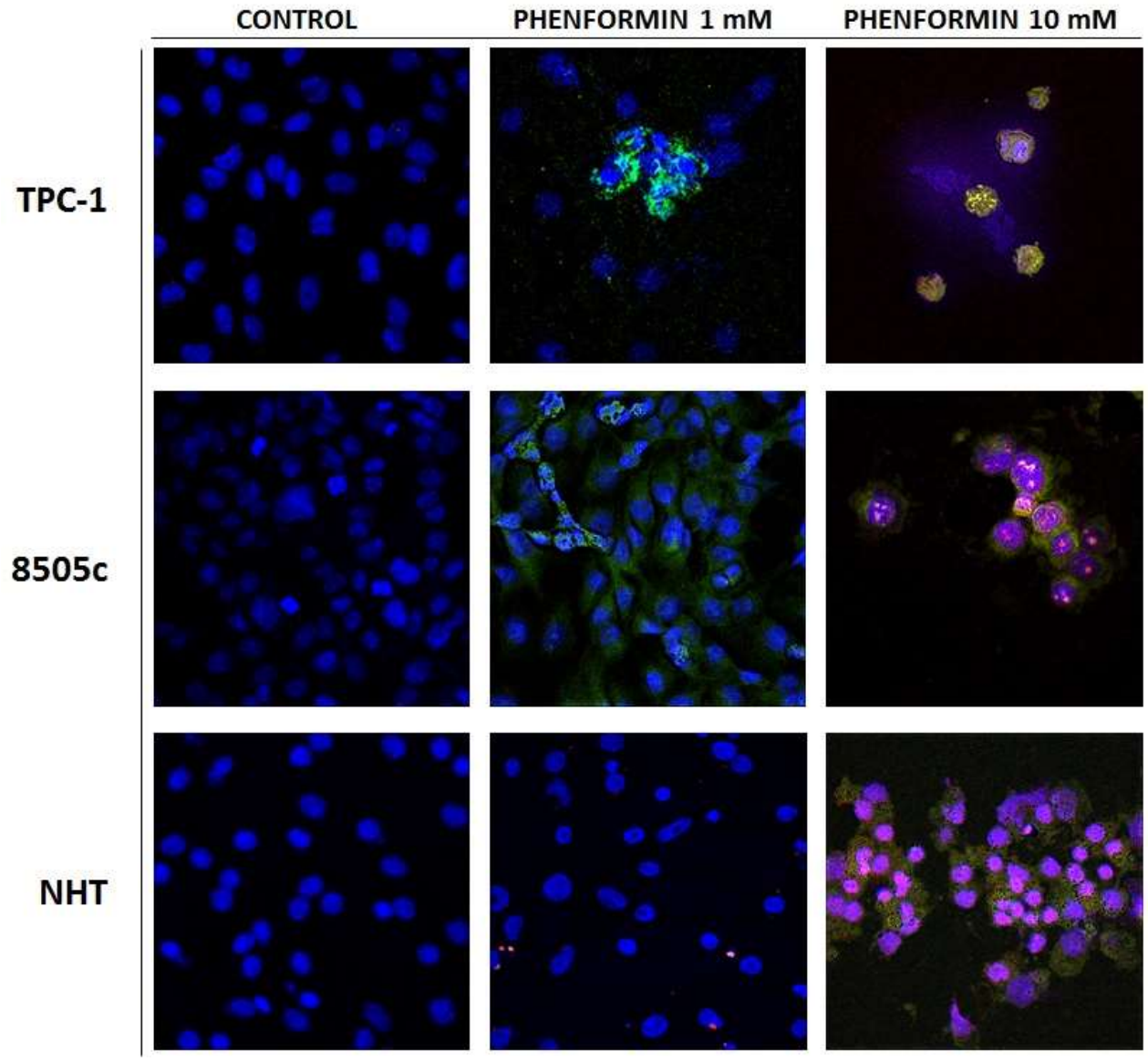

Figure 5

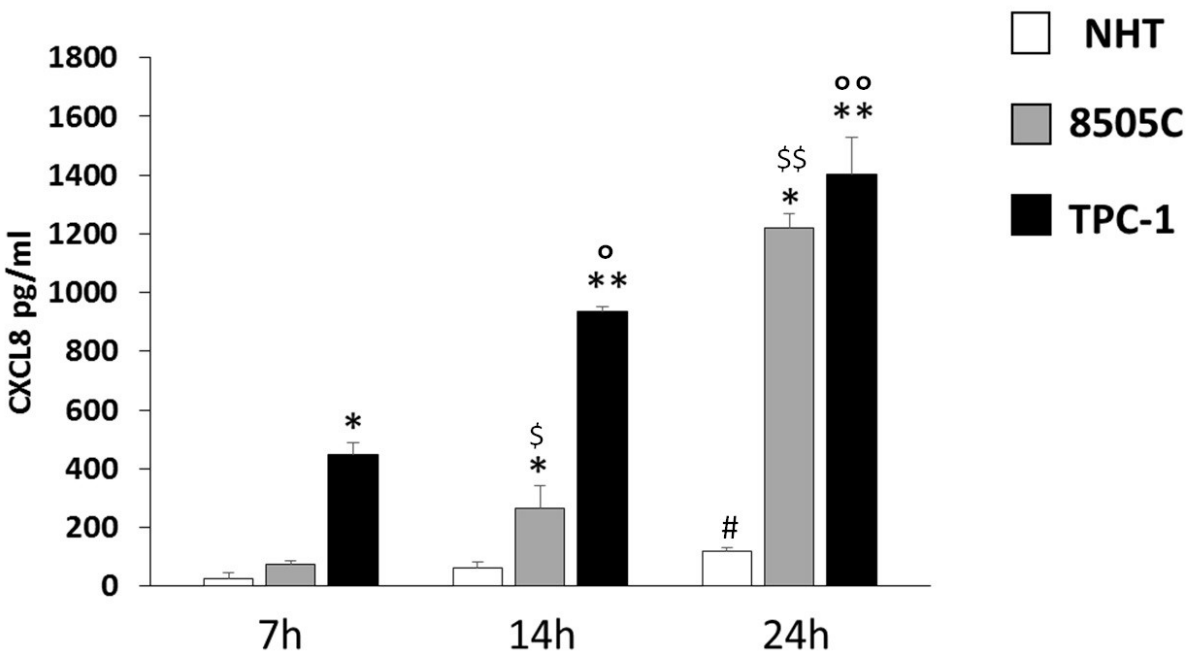
1800
1600
1400
1200
1000
800
600
400
200
0
CXCL8 pg/ml
7h
14h
24h
NHT
8505C
TPC-1
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Figure 6

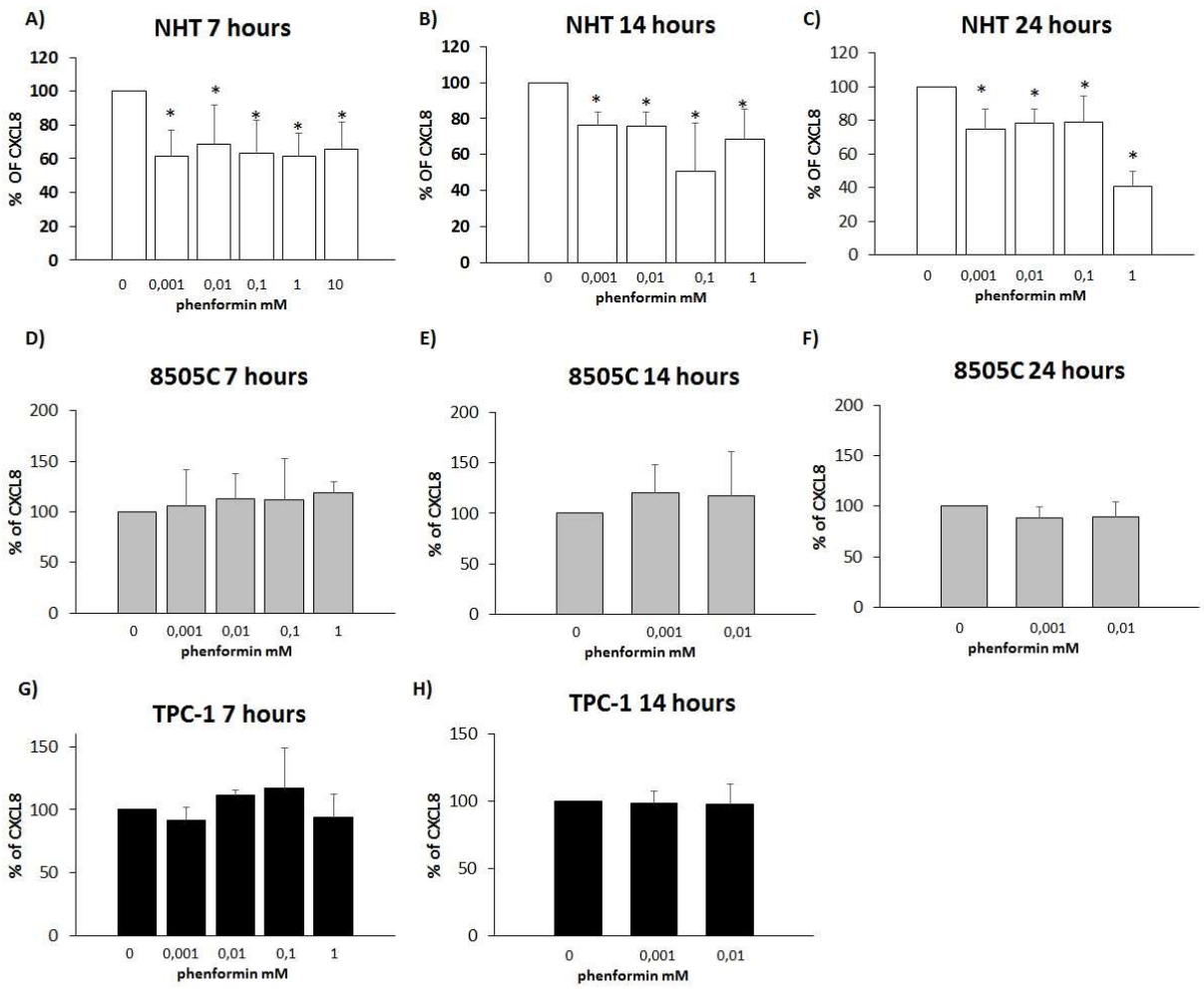
A)
NHT 7 hours
% OF CXCL8
phenformin mM
B)
NHT 14 hours
% OF CXCL8
phenformin mM
C)
NHT 24 hours
% OF CXCL8
phenformin mM
D)
8505C 7 hours
% of CXCL8
phenformin mM
E)
8505C 14 hours
% of CXCL8
phenformin mM
F)
8505C 24 hours
% of CXCL8
phenformin mM
G)
TPC-1 7 hours
% of CXCL8
phenformin mM
H)
TPC-1 14 hours
% of CXCL8
phenformin mM

Figure 7

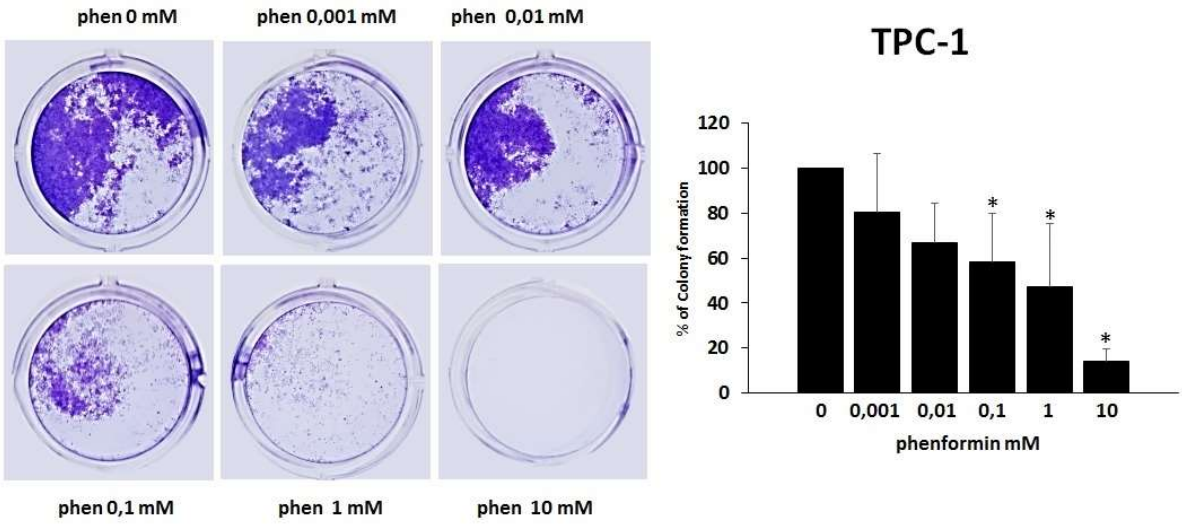

Figure 8

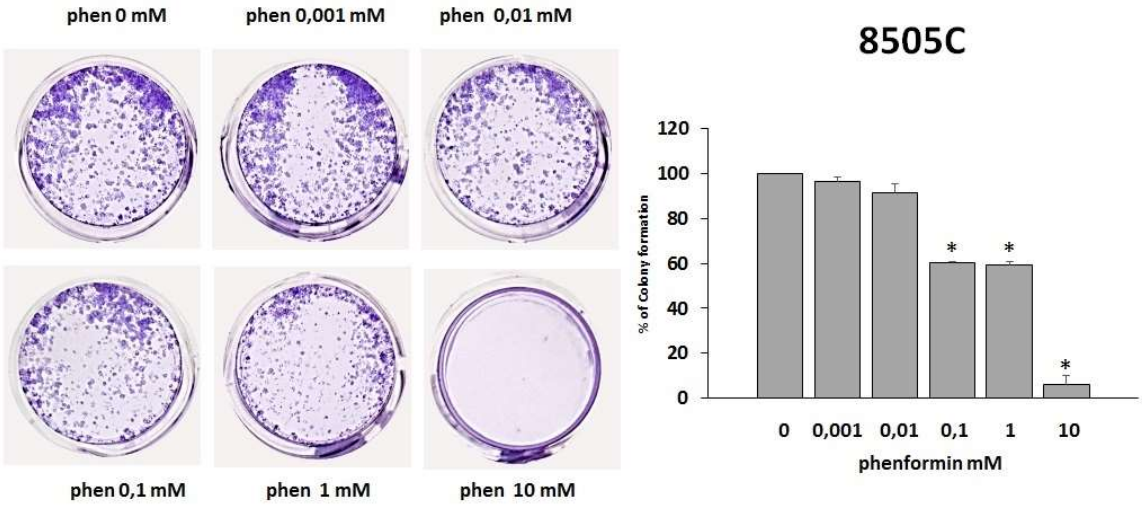

Figure 9

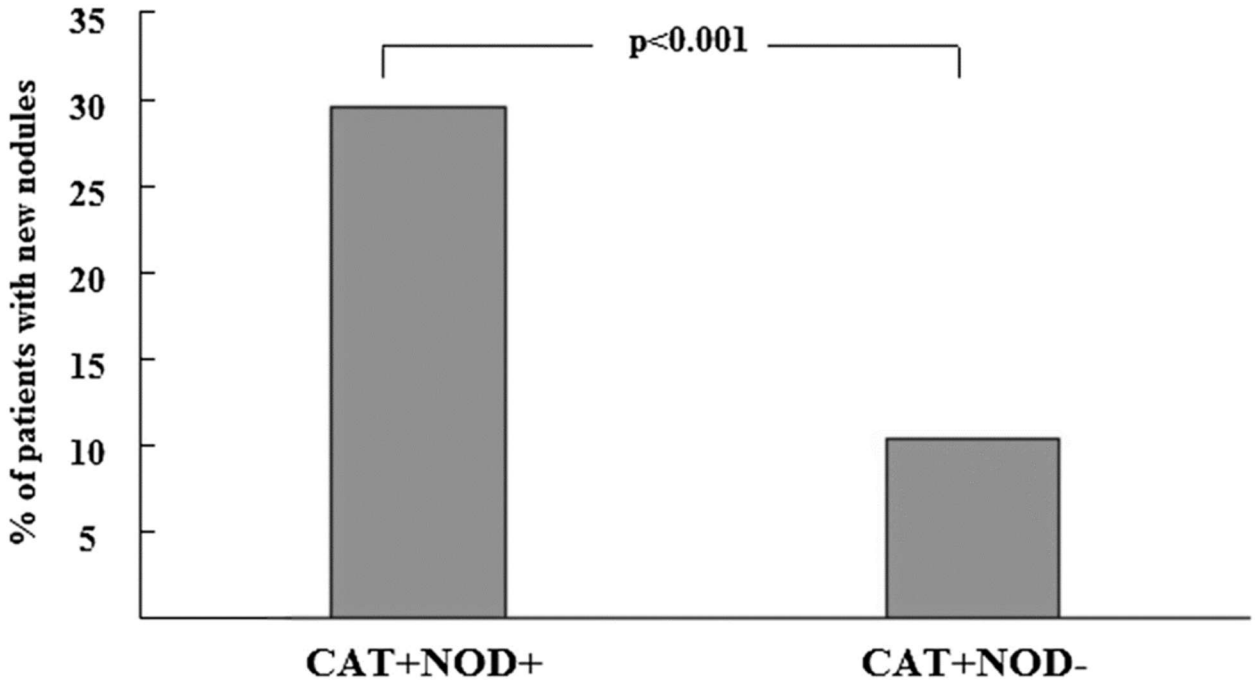
35
30
25
20
15
10
5
% of patients with new nodules
p<0.001
CAT+NOD+
CAT+NOD-

Figure 10

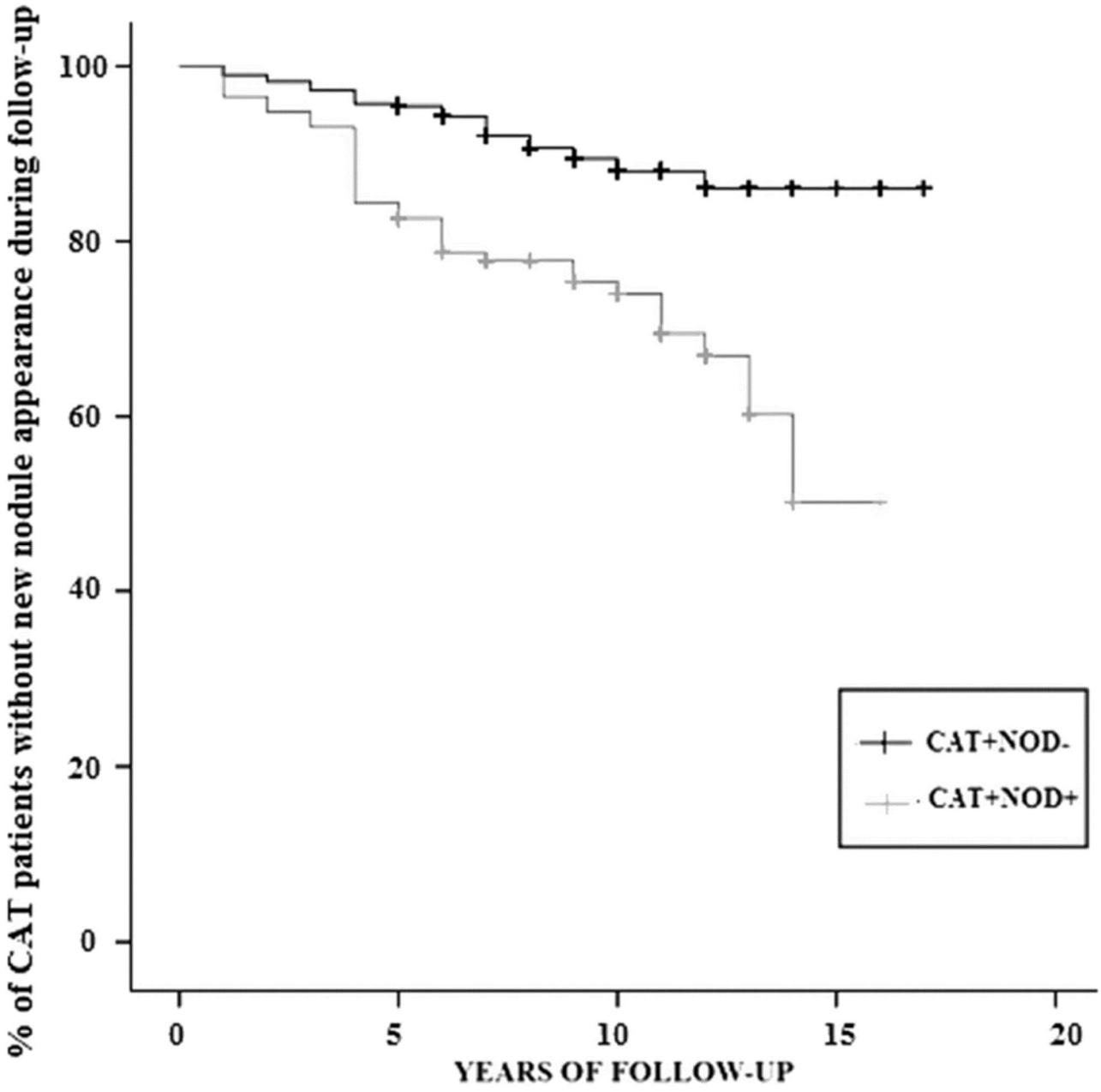
% of CAT patients without new nodule appearance during follow-up
100
80
60
40
20
0
0
5
10
15
20
YEARS OF FOLLOW-UP
CAT+NOD-
CAT+NOD+

Figure 11

FIGURE LEGENDS

Figure 1: Panel A: PLX4720 inhibited the basal CXCL8 secretion in BCPAP (ANOVA F: 14.3; p<0.0001), the inhibitory effect was significant starting by 2μM concentration (Post Hoc analysis by Bonferroni *p<0.001 vs. basal). **Panel B:** PLX4720 inhibited the basal CXCL8 secretion in 8305C (ANOVA F: 407.9; p<0.0001) (ANOVA F: 407.9; p<0.0001), the inhibitory effect was significant starting from 0.1 μM (Post Hoc analysis by Bonferroni *p<0.001 vs. basal, **p<0.001 vs. 0,1μM). **Panel C:** PLX4720 inhibited the basal CXCL8 secretion in 8505C (ANOVA F: 55.24; p<0.0001), the inhibitory effect started from 0.1 μM (Post Hoc analysis by Bonferroni *p<0.001 vs. basal, p<0.001 vs. 0.1 μM). **Panel D:** Basal secretion of CXCL8 was not inhibited by PLX4720 in TPC-1 cell lines at any concentrations (ANOVA F: 1.8, p=1.34). **Panel E:** Basal secretion of CXCL8 was inhibited by PLX4720 in NHT (ANOVA F: 13.13; p<0.001) being significant only at the higher concentration of 10μM (*Post Hoc* analysis by Bonferroni *p<0.01 *vs.* basal). **Panel F:** PLX4720 inhibit the TNF-α-stimulated CXCL8 secretion in BCPAP (ANOVA F: 12.29 p<0.0001), the inhibitory effect was significant starting by 1 10μM (3.13; t an*Post Hoc* analysis by Bonferroni *p<0.001 vs. TNF-α). **Panel G:** PLX4720 inhibit the TNF-α-stimulated CXCL8 secretion in 8305C (ANOVA F: 5.76 p<0.0001), the inhibitory effect started from 1 rom *Post Hoc* analysis by Bonferroni *p<0.001 vs. TNF-α). **Panel H:** PLX4720 inhibit the TNF-α-stimulated CXCL8 secretion in 8505C (ANOVA F: 42.85 p<0.0001), the inhibitory effect started from 0.1 rom *Post Hoc* analysis by Bonferroni *p<0.001 vs. TNF-α, **p<0.001 vs. 0.1 ni *p< **Panel I:** TNF-α-stimulated CXCL8 secretion was not inhibited by PLX4720 in TPC-1 cell lines at any concentrations (ANOVA F:1.6, p=1.78). **Panel E:** TNF-α-stimulated CXCL8 secretion was inhibited by PLX4720 in NHT (ANOVA F: 2.5; p<0.01) being significant only at the higher concentration of10μM (*Post Hoc* analysis by Bonferroni *p<0.001 *vs.* TNF-α alone).

Figure 2: CXCL8 concentrations progressively increased throughout the 72-h time course in untreated thyroid cells. **Panel A:** BCPAP cells (ANOVA: F=13.39; p<0.0001). **Panel B**: NHT cells (ANOVA: F=2,7; p<0.002). **Panel C:** 8305C cells (ANOVA F=6.76; p<0.009). **Panel D:** 8505C cells (ANOVA: F=11.09; p<0.001)

Figure 3: Time course of PLX4720 inhibitory effect on CXCL8 secretion. No significant change in the inhibitory power of PLX4720 at a given concentration was found throughout the 72-h time course. **Panel A:** BCPAP (ANOVAs: PLX4720 0.1μM F: 0.6; NS; 1μM F: 1.7; NS; 2μM F: 1.7; p= 0.2; 5μM F: 2.7; p= 0.7; 10 μM F: 1.0, NS). **Panel B:** NHT (ANOVAs: PLX4720 0.1μM F:1.7, NS; 1μM F:2.8; NS; 2μM F: 3; NS; 5μM F: 1; NS; 10 μM F: 2.3, NS).**Panel C:** 8305C (ANOVAs: PLX4720 0.1μM F:1.6, NS; 1μM F: 2.6; NS; 2μM F: 0.5; NS; 5μM F: 0.4; NS; 10 μM F: 1.3, NS). **Panel D:** 8505C (ANOVAs: PLX4720 0.1μM F:0.02, NS; 1μM F: 0.2; NS; 2μM F: 0.1; NS; 5μM F: 1.2; NS; 10 μM F: 2.9, NS).

Figure 4: Effect of PLX4720 on the basal and rh-CXCL8-induced migration in BCPAP, 8305C, 8505C, NHT and TPC-1 thyroid cancer cells. Representative images and the respective histograms after 16 hours of migration within the trans-well migration chamber system in BCPAP, 8305C, 8505C, NHT and TPC-1 (Panels A, B, C, D, E respectively). **Panel A)** in BCPAP the treatment with PLX4720 10μM reduced the basal migration (ANOVA F= 27.9, p<0.0001; *Post Hoc* ° p<0.005 vs. basal). The incubation of BCPAP with rh-CXCL8 produced a significant increase of BCPAP migration (*Post Hoc* *p<0.0001 vs. basal). The co-incubation with rh-CXCL8 and PLX4720 10μM significantly inhibited the BCPAP migration induced by rh-CXCL8 (*Post Hoc* # p<0.001 vs. CXCL8). **Panel B)** in 8305C, the treatment with PLX4720 10μM reduced the basal migration of 8305C (ANOVA F= 161.7 , p<0.0001; *Post Hoc* ° p<0.005 vs. basal). The incubation of 8305C with rh-CXCL8 produced a significant increase of 8305C migration (*Post Hoc* *p<0.0001 vs. basal). The co-incubation with rh-CXCL8 and PLX4720 10μM significantly inhibited the 8305C migration induced by rh-CXCL8 (*Post Hoc* # p<0.0001 vs. CXCL8). **Panel C)** in 8505C the treatment with

PLX4720 10μM reduced the basal migration of 8505C (ANOVA F= 21.6 , p<0.0001; *Post Hoc* °p<0.005 vs. basal). The incubation of 8505C with rh-CXCL8 produced a significant increase of 8505C migration (*Post Hoc* *p<0.0001 vs. basal). The co-incubation with rh-CXCL8 and PLX4720 10μM significantly inhibited the 8505C migration induced by rh-CXCL8 (*Post Hoc* # p<0.0001 vs. CXCL8). **Panel D)** in NHT, the treatment with PLX4720 10μM reduced the basal migration of NHT (ANOVA F= 25.3 , p<0.0001; *Post Hoc* ° p<0.005 vs. basal). The incubation of NHT with rh-CXCL8 produced a significant increase of NHT migration (*Post Hoc* *p<0.05 vs. basal). The co-incubation with rh-CXCL8 and PLX4720 10μM significantly inhibited the NHT migration induced by rh-CXCL8 (*Post Hoc* # p<0.0001 vs. CXCL8).**Panel E)** in TPC-1 the treatment with PLX4720 10μM did not reduce the basal migration of TPC-1 (ANOVA F= 4.4 , p<0.01), *Post Hoc* NS vs. basal). The incubation of TPC-1 with rh-CXCL8 produced a significant increase of TPC-1 migration (*Post Hoc* *p<0.05 vs. basal). The co-incubation with rh-CXCL8 and PLX4720 10μM did not reduce the TPC-1 migration induced by rh-CXCL8 (*Post Hoc* # NS vs. CXCL8). Bar graphs show the corresponding analysis of migrated cells on the lower side of the transwell filter. Basal migration was conventionally estimated as 100%.

Figure 5: Cell viability of TPC-1, 8505C and NHT after treatment with Phenformin at increasing concentrations at different time points.

Panel A) Phenformin 10mM reduces TPC-1 viability after 7 hours of cell (ANOVA F=3.765; p<0.005). Panel B) Phenformin 10 mM reduces cell viability after 14 hours of cell incubation (ANOVA F=12.160; p<0.001). Panel C) Phenformin reduces cell viability at all concentration tested after 24 hours of cell incubation (ANOVA F=42.537; p<0.001). Panel D) Phenformin 10mM reduces 8505C viability after 7 hours of cell (ANOVA F=3.482; p<0.05). Panel E) Phenformin reduces cell viability after 14 hours of cell incubation starting from 0.1 mM (ANOVA F=15.007; p<0.001). Panel F) Phenformin reduces cell viability after 24 hours of cell incubation starting from 0.1 mM (ANOVA

F=10.129; p<0.001). Panel G) Phenformin does not affect NHT cells viability after 7 hours (ANOVA NS). Panel H) Phenformin 10 mM reduces cell viability after 14 hours of cell incubation (ANOVA: F=8.892: p<0.001). Panel I) Phenformin reduces cell viability at all concentration tested after 24 hours of cell incubation (ANOVA F=12.7; p<0.001). IC50 values are shown for 24h treatment with phenformin of TPC-1 and 8505C.

*Post Hoc analysis by Bonferroni p<0.05 vs. basal.

Figure 6: Apoptosis induced in treated thyroid cancer cell lines observed with Annexin V-FITC/PI stain under confocal fluorescent microscope (40× magnification). These are representative of merged Annexin V/PI/Hoecst images of three experiments. The control untreated cells appear positive only for hoecst staining of the nuclei. The fluorescent bright orange-green stain of Annexin V-FITC/PI in treated cells demonstrate apoptosis. The PI stain binds to the damaged nucleus of the treated cancer cells. In particular these image evidence that both TPC-1 and 8505C thyroid cancer cell lines are positive for annexin V after treatment with phenformin 1 mM, In addition thyroid cancer cell lines are positive for AnnexinV and PI after treatment with pjhenformin 10mM. On the other hand NHT cells results positive for AnnexinV/PI staining only after treatment with phenformin 10 mM, while are negative for treatment with phenformin 1mM.

Figure 7: CXCL8 increase in NHT 8505C and TPC-1 throughout the time course. NHT (ANOVA F=4.5 p<0.05; Post Hoc 24h p<0.05 vs. 7h), 8505C (ANOVA F=512.26 p<0.001; Post Hoc 24h p<0.05 vs. 14h and 7h, 14h p<0.05 vs. 7h), TPC-1 (ANOVA F=158.72 p<0.001; Post Hoc 24h p<0.05 vs. 14h and 7h, 14h p<0.05 vs. 7h). Panel D) the secretion of CXCL8 differs among the cell types at 7 14 and 24 hours being TPC-1>8505C>NHT. After a 7-hour incubation period CXCL8 levels were higher in TPC-1 supernatants as compared with the NHT and 8505C ones (ANOVA F=218.43

p<0.001; Post Hoc TPC-1 p<0.05 vs. 8505C and NHT). After 14 hours of incubation, TPC-1 cell again secreted the greatest amounts of CXCL8, followed by 8505C cells, which secreted higher levels as compared with NHT cells (TPC-1>8505C>NHT) (ANOVA F=332.78 p<0.001; Post Hoc TPC-1 p<0.05 vs. 8505C and NHT, 8505C p<0.05 vs. NHT). A similar secretion gradient was observed after 24 hours: TPC-1 > 8505C > NHT cells (ANOVA F=325.742 p<0.001; Post Hoc TPC-1 p<0.05 vs. 8505C and NHT, 8505C p<0.05 vs. NHT). *p<0,05 vs. NHT ; ** p<0,05 vs. 8505C and NHT; # p<0,05 vs. 7h in NHT; $ p<0,05 vs. 7h in 8505C; $$ p<0,05 vs. 7h and 14h in 8505C; ° p<0,05 vs. 7h in TPC-1; °°p<0,05 vs. 7h and 14h in TPC-1.

Figure 8: Inhibition of CXCL8 secretion. Panel A) Phenformin inhibits CXCL8 secretion at all non-cytotoxic concentrations after 7 hour of treatment in NHT (ANOVA F=5.702; p<0.001) Panel B) Phenformin inhibits CXCL8 secretion at all non-cytotoxic concentrations after 14 hour of treatment in NHT (ANOVA F=11.15; p<0.001) Panel C) Phenformin inhibits CXCL8 secretion at all non-cytotoxic concentrations after 24 hours of treatment in NHT (ANOVA F=24.819; p<0.001). Panel D) Phenformin does not inhibit CXCL8 secretion after 7 hours of treatment in 8505C cells (ANOVA not significant) Panel E) Phenformin does not inhibit CXCL8 secretion after 14 hours of treatment in 8505C cells (ANOVA not significant) Panel F) Phenformin does not inhibit CXCL8 secretion after 14 hours of treatment in 8505C cells (ANOVA not significant) Panel G) Phenformin does not inhibit CXCL8 secretion after 7 hours of treatment in TPC-1 cells (ANOVA not significant) Panel H) Phenformin does not inhibit CXCL8 secretion after 14 hours of treatment in TPC-1 cells (ANOVA not significant)

*Post Hoc analysis by Bonferroni p<0.05 vs. basal.

Figure 9: Effects of phenformin on TPC-1 cells ability to form colonies. Colony formation assay was used to determine the long-term effects of phenformin on cell proliferation. The picture is one representative experiment of the results obtained. As indicated by histogram colony formation is reduced by phenformin treatment (ANOVA F=9.068 p< 0.0001) starting from 0.1mM concentration, as assessed by optical density measurement after staining with Crystal violet and incubation with SDS. Colony formation is expressed as a percentage of the untreated control.

Post hoc *p < 0.05, statistically significant.

Figure 10: Effects of phenformin on 8505C cells ability to form colonies. Colony formation assay was used to determine the long-term effects of phenformin on cell proliferation. The picture is one representative experiment of the results obtained. As indicated by histogram colony formation is reduced by phenformin treatment ANOVA F=311.75 p<0.0001) starting from 0.1mM concentration, as assessed by optical density measurement after staining with Crystal violet and incubation with SDS. Colony formation is expressed as a percentage of the untreated control.

Post hoc *p < 0.05, statistically significant.

Figure 11: Percentage of patients who developed new nodules during the follow-up.

Figure 12. Survival analysis for the development of new nodules in the CAT+ NOD+ and CAT+ NOD- subgroups. (Kaplan-Meier Log Rank 24.441, p<0.001).

TABLES

	CAT+, NOD+ (n 115)	CAT+, NOD- (n 395)	p value
M/F	7/108	27/368	0.486
Age (years) Median (range)	52 (21-81)	48 (19-82)	< 0.001
Thyroid volume (ml) Median (Range)	13 (4-64)	10 (2-70)	< 0.001
Serum TSH at study entry (median, 5^{th}-95^{th} centiles)	2.4 (0.6-11.37)	4.38 (0.73-38.0)	<0.001
Serum TSH at the last evaluation (median, 5th-95th centiles)	1.76 (0.50-5.72)	2.14 (0.60-6.57)	<0.05
Thyroid Antibody Status TgAb+/TPOAb+ TgAb+/TPOAb- TgAb-/TPOAb+ TgAb-/TPOAb-	 62 (53.9%) 13 (11.3%) 31 (27.0%) 9 (7.9%)	 235 (59.5%) 31 (7.8%) 103 (26.1%) 26 (6.6%)	 0.586

Table 1: Demographic data, thyroid function and thyroid autoantibody status, and US features in the CAT+ NOD + and CAT+ NOD - groups.

	CAT+, NOD+ (n 115)	**CAT+, NOD- (n 395)**	***p* value**
Follow-up time (years) (median and range)	11 (5-16)	10 (5-17)	0.083
N. of patients developing neo nodules (%)	34/115 (29.5%)	41/395 (10.3%)	<0.001
Nodules not requiring FNAC (%)	20/34 (58.8%)	28/41(68.3%)	0.472
Maximum Diameter (mm)	7.5±1.6	6.6±2.0	0.108
EUTIRADS 3 4	 13/20 (65.0%) 7/20 (35.0%)	 22/28 (78.6%) 6/28 (21.4%)	 0.297
Nodules requiring FNAC (%)	14/34 (41.2%)	13/41 (31.7%)	0.472
Maximum Diameter (mm)	13.2±3.7	12.8±4.3	0.831
EUTIRADS 3 4 5	 9/14 (64.3%) 3/14 (21.4%) 2/14 (14.3%)	 8/13 (51.5%) 3/13 (23.1%) 2/13 (15.4%)	 0.989
Benign Cytology	14/14 (100%)	13/13 (100%)	0.989

Table 2: Appearance of new nodules in CAT+NOD+ and CAT+NOD- groups and corresponding Ultrasound and FNAC results

	p value	Exp(B)	95% CI fo Exp(B)	
			Lower	Upper
Age (years)	0.532	0.994	0.975	1.013
Gender (M/F)	0.674	0.797	0.276	2.298
Thyroid Volume (ml)	**0.028**	1.030	1.003	1.057
Group (CATNOD-/CATNOD+)	**<0.001**	3.337	1.942	5.734

Table 3. Logistic Regression analysis for assessing the risk of new nodule development

REFERENCES

1. Islami F, Ward EM, Sung H, Cronin KA, Tangka FKL, Sherman RL, Zhao J, Anderson RN, Henley SJ, Yabroff KR, Jemal A, Benard VB. Annual Report to the Nation on the Status of Cancer, Part 1: National Cancer Statistics. J Natl Cancer Inst. 2021.

2. Nilubol N, Zhang L, Kebebew E. Multivariate analysis of the relationship between male sex, disease-specific survival, and features of tumor aggressiveness in thyroid cancer of follicular cell origin. Thyroid. 2013;23(6):695-702.

3. Kim J, Gosnell JE, Roman SA. Geographic influences in the global rise of thyroid cancer. Nat Rev Endocrinol. 2020;16(1):17-29.

4. Molinaro E, Romei C, Biagini A, Sabini E, Agate L, Mazzeo S, Materazzi G, Sellari-Franceschini S, Ribechini A, Torregrossa L, Basolo F, Vitti P, Elisei R. Anaplastic thyroid carcinoma: from clinicopathology to genetics and advanced therapies. Nat Rev Endocrinol. 2017;13(11):644-660.

5. Filetti S, Durante C, Hartl D, Leboulleux S, Locati LD, Newbold K, Papotti MG, Berruti A, clinicalguidelines@esmo.org EGCEa. Thyroid cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up†. Ann Oncol. 2019;30(12):1856-1883.

6. Asioli S, Erickson LA, Sebo TJ, Zhang J, Jin L, Thompson GB, Lloyd RV. Papillary thyroid carcinoma with prominent hobnail features: a new aggressive variant of moderately differentiated papillary carcinoma. A clinicopathologic, immunohistochemical, and molecular study of eight cases. Am J Surg Pathol. 2010;34(1):44-52.

7. Aschebrook-Kilfoy B, Ward MH, Sabra MM, Devesa SS. Thyroid cancer incidence patterns in the United States by histologic type, 1992-2006. Thyroid. 2011;21(2):125-134.

8. Zeng Z, Li K, Kang W, Yu J, Wang X, Zhang Z, Sun J, Ouyang S. Changing patterns of thyroid cancer in different stages of Universal Salt Iodization in Peking Union Medical College Hospital, 1986-2018. Gland Surg. 2020;9(5):1338-1345.

9. Amendoeira I, Maia T, Sobrinho-Simões M. Non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP): impact on the reclassification of thyroid nodules. Endocr Relat Cancer. 2018;25(4):R247-R258.

10. Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, Davis N, Dicks E, Ewing R, Floyd Y, Gray K, Hall S, Hawes R, Hughes J, Kosmidou V, Menzies A, Mould C, Parker A, Stevens C, Watt S, Hooper S, Wilson R, Jayatilake H, Gusterson BA, Cooper C, Shipley J, Hargrave D, Pritchard-Jones K, Maitland N, Chenevix-Trench G, Riggins GJ, Bigner DD, Palmieri G, Cossu A, Flanagan A, Nicholson A, Ho JW, Leung SY, Yuen ST, Weber BL, Seigler HF, Darrow TL, Paterson H, Marais R, Marshall CJ, Wooster R, Stratton MR, Futreal PA. Mutations of the BRAF gene in human cancer. Nature. 2002;417(6892):949-954.

11. Caronia LM, Phay JE, Shah MH. Role of BRAF in thyroid oncogenesis. Clin Cancer Res. 2011;17(24):7511-7517.

12. Borrello MG, Alberti L, Fischer A, Degl'innocenti D, Ferrario C, Gariboldi M, Marchesi F, Allavena P, Greco A, Collini P, Pilotti S, Cassinelli G, Bressan P, Fugazzola L, Mantovani A, Pierotti MA. Induction of a proinflammatory program in normal human thyrocytes by the RET/PTC1 oncogene. Proc Natl Acad Sci U S A. 2005;102(41):14825-14830.

13. Fusco A, Grieco M, Santoro M, Berlingieri MT, Pilotti S, Pierotti MA, Della Porta G, Vecchio G. A new oncogene in human thyroid papillary carcinomas and their lymph-nodal metastases. Nature. 1987;328(6126):170-172.

14. Howell GM, Hodak SP, Yip L. RAS mutations in thyroid cancer. Oncologist. 2013;18(8):926-932.

15. Davies L, Welch HG. Increasing incidence of thyroid cancer in the United States, 1973-2002. JAMA. 2006;295(18):2164-2167.

16. Ahn HS, Welch HG. South Korea's Thyroid-Cancer "Epidemic"--Turning the Tide. N Engl J Med. 2015;373(24):2389-2390.

17. Grodski S, Brown T, Sidhu S, Gill A, Robinson B, Learoyd D, Sywak M, Reeve T, Delbridge L. Increasing incidence of thyroid cancer is due to increased pathologic detection. Surgery. 2008;144(6):1038-1043; discussion 1043.

18. Dal Maso L, Panato C, Franceschi S, Serraino D, Buzzoni C, Busco S, Ferretti S, Torrisi A, Falcini F, Zorzi M, Cirilli C, Mazzucco W, Magoni M, Collarile P, Pannozzo F, Caiazzo AL, Russo AG, Gili A, Caldarella A, Zanetti R, Michiara M, Mangone L, Filiberti RA, Fusco M, Gasparini F, Tagliabue G, Cesaraccio R, Tumino R, Gatti L, Tisano F, Piffer S, Sini GM, Mazzoleni G, Rosso S, Fanetti AC, Vaccarella S, group fAw. The impact of overdiagnosis on thyroid cancer epidemic in Italy,1998-2012. Eur J Cancer. 2018;94:6-15.

19. Haugen BR. 2015 American Thyroid Association Management Guidelines for Adult Patients with Thyroid Nodules and Differentiated Thyroid Cancer: What is new and what has changed? Cancer. 2017;123(3):372-381.

20. Haugen BR, Sawka AM, Alexander EK, Bible KC, Caturegli P, Doherty GM, Mandel SJ, Morris JC, Nassar A, Pacini F, Schlumberger M, Schuff K, Sherman SI, Somerset H, Sosa JA, Steward DL, Wartofsky L, Williams MD. American Thyroid Association Guidelines on the Management of Thyroid Nodules and Differentiated Thyroid Cancer Task Force Review and Recommendation on the Proposed Renaming of Encapsulated Follicular Variant Papillary Thyroid Carcinoma Without Invasion to Noninvasive Follicular Thyroid Neoplasm with Papillary-Like Nuclear Features. Thyroid. 2017;27(4):481-483.

21. Mazonakis M, Tzedakis A, Damilakis J, Gourtsoyiannis N. Thyroid dose from common head and neck CT examinations in children: is there an excess risk for thyroid cancer induction? Eur Radiol. 2007;17(5):1352-1357.

22. Baker SR, Bhatti WA. The thyroid cancer epidemic: is it the dark side of the CT revolution? Eur J Radiol. 2006;60(1):67-69.

23. Marotta V, Russo G, Gambardella C, Grasso M, La Sala D, Chiofalo MG, D'Anna R, Puzziello A, Docimo G, Masone S, Barbato F, Colao A, Faggiano A, Grumetto L. Human exposure to bisphenol AF and diethylhexylphthalate increases susceptibility to develop differentiated thyroid cancer in patients with thyroid nodules. Chemosphere. 2019;218:885-894.

24. Pellegriti G, De Vathaire F, Scollo C, Attard M, Giordano C, Arena S, Dardanoni G, Frasca F, Malandrino P, Vermiglio F, Previtera DM, D'Azzò G, Trimarchi F, Vigneri R. Papillary thyroid cancer incidence in the volcanic area of Sicily. J Natl Cancer Inst. 2009;101(22):1575-1583.

25. Kitahara CM, Pfeiffer RM, Sosa JA, Shiels MS. Impact of Overweight and Obesity on US Papillary Thyroid Cancer Incidence Trends (1995-2015). J Natl Cancer Inst. 2020;112(8):810-817.

26. Fussey JM, Beaumont RN, Wood AR, Vaidya B, Smith J, Tyrrell J. Does Obesity Cause Thyroid Cancer? A Mendelian Randomization Study. J Clin Endocrinol Metab. 2020;105(7).

27. Trimboli P, Ngu R, Royer B, Giovanella L, Bigorgne C, Simo R, Carroll P, Russ G. A multicentre validation study for the EU-TIRADS using histological diagnosis as a gold standard. Clin Endocrinol (Oxf). 2019;91(2):340-347.

28. Grant EG, Tessler FN, Hoang JK, Langer JE, Beland MD, Berland LL, Cronan JJ, Desser TS, Frates MC, Hamper UM, Middleton WD, Reading CC, Scoutt LM, Stavros AT, Teefey SA. Thyroid Ultrasound Reporting Lexicon: White Paper of the ACR Thyroid Imaging, Reporting and Data System (TIRADS) Committee. J Am Coll Radiol. 2015;12(12 Pt A):1272-1279.

29. Middleton WD, Teefey SA, Reading CC, Langer JE, Beland MD, Szabunio MM, Desser TS. Multiinstitutional Analysis of Thyroid Nodule Risk Stratification Using the American College of Radiology Thyroid Imaging Reporting and Data System. AJR Am J Roentgenol. 2017;208(6):1331-1341.

30. Castellana M, Castellana C, Treglia G, Giorgino F, Giovanella L, Russ G, Trimboli P. Performance of five ultrasound risk stratification systems in selecting thyroid nodules for FNA. A meta-analysis. J Clin Endocrinol Metab. 2019.

31. Cibas ES, Ali SZ. The Bethesda System for Reporting Thyroid Cytopathology. Thyroid. 2009;19(11):1159-1165.

32. Maletta F, Massa F, Torregrossa L, Duregon E, Casadei GP, Basolo F, Tallini G, Volante M, Nikiforov YE, 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.

33. Nikiforova MN, Mercurio S, Wald AI, Barbi de Moura M, Callenberg K, Santana-Santos L, Gooding WE, Yip L, Ferris RL, Nikiforov YE. Analytical performance of the ThyroSeq v3 genomic classifier for cancer diagnosis in thyroid nodules. Cancer. 2018;124(8):1682-1690.

34. Ito Y, Miyauchi A, Oda H. Low-risk papillary microcarcinoma of the thyroid: A review of active surveillance trials. Eur J Surg Oncol. 2018;44(3):307-315.

35. Hauch A, Al-Qurayshi Z, Randolph G, Kandil E. Total thyroidectomy is associated with increased risk of complications for low- and high-volume surgeons. Ann Surg Oncol. 2014;21(12):3844-3852.

36. Adam MA, Pura J, Gu L, Dinan MA, Tyler DS, Reed SD, Scheri R, Roman SA, Sosa JA. Extent of surgery for papillary thyroid cancer is not associated with survival: an analysis of 61,775 patients. Ann Surg. 2014;260(4):601-605; discussion 605-607.

37. Goldman JM, Goren EN, Cohen MH, Webber BL, Brennan MF, Robbins J. Anaplastic thyroid carcinoma: long-term survival after radical surgery. J Surg Oncol. 1980;14(4):389-394.

38. Pacini F, Ladenson PW, Schlumberger M, Driedger A, Luster M, Kloos RT, Sherman S, Haugen B, Corone C, Molinaro E, Elisei R, Ceccarelli C, Pinchera A, Wahl RL, Leboulleux S, Ricard M, Yoo J, Busaidy NL, Delpassand E, Hanscheid H, Felbinger R, Lassmann M, Reiners C. Radioiodine ablation of thyroid remnants after preparation with recombinant human thyrotropin in differentiated thyroid carcinoma: results of an international, randomized, controlled study. J Clin Endocrinol Metab. 2006;91(3):926-932.

39. Lamartina L, Durante C, Filetti S, Cooper DS. Low-risk differentiated thyroid cancer and radioiodine remnant ablation: a systematic review of the literature. J Clin Endocrinol Metab. 2015;100(5):1748-1761.

40. Durante C, Attard M, Torlontano M, Ronga G, Monzani F, Costante G, Ferdeghini M, Tumino S, Meringolo D, Bruno R, De Toma G, Crocetti U, Montesano T, Dardano A, Lamartina L, Maniglia A, Giacomelli L, Filetti S, Group PTCS. Identification and optimal postsurgical follow-up of patients with very low-risk papillary thyroid microcarcinomas. J Clin Endocrinol Metab. 2010;95(11):4882-4888.

41. Tuttle RM, Ahuja S, Avram AM, Bernet VJ, Bourguet P, Daniels GH, Dillehay G, Draganescu C, Flux G, Führer D, Giovanella L, Greenspan B, Luster M, Muylle K, Smit JWA, Van Nostrand D, Verburg FA, Hegedüs L. Controversies, Consensus, and Collaboration in the Use of. Thyroid. 2019;29(4):461-470.

42. Haugen BR, Alexander EK, Bible KC, Doherty GM, Mandel SJ, Nikiforov YE, Pacini F, Randolph GW, Sawka AM, Schlumberger M, Schuff KG, Sherman SI, Sosa JA, Steward DL, Tuttle RM, Wartofsky L. 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):1-133.

43. Osorio M, Moubayed SP, Su H, Urken ML. Systematic review of site distribution of bone metastases in differentiated thyroid cancer. Head Neck. 2017;39(4):812-818.

44. Schlumberger M, Brose M, Elisei R, Leboulleux S, Luster M, Pitoia F, Pacini F. Definition and management of radioactive iodine-refractory differentiated thyroid cancer. Lancet Diabetes Endocrinol. 2014;2(5):356-358.

45. Wexler JA. Approach to the thyroid cancer patient with bone metastases. J Clin Endocrinol Metab. 2011;96(8):2296-2307.

46. Drost L, Ganesh V, Wan BA, Raman S, Chan S, Christakis M, Tsao M, Barnes E, Ford M, Finkelstein J, Yee A, Turner A, Lam H, Chow E. Efficacy of postoperative radiation treatment for bone metastases in the extremities. Radiother Oncol. 2017;124(1):45-48.

47. Deschamps F, Farouil G, de Baere T. Percutaneous ablation of bone tumors. Diagn Interv Imaging. 2014;95(7-8):659-663.

48. Brose MS, Nutting CM, Jarzab B, Elisei R, Siena S, Bastholt L, de la Fouchardiere C, Pacini F, Paschke R, Shong YK, Sherman SI, Smit JW, Chung J, Kappeler C, Peña C, Molnár I, Schlumberger MJ, investigators D. Sorafenib in radioactive iodine-refractory, locally advanced or metastatic differentiated thyroid cancer: a randomised, double-blind, phase 3 trial. Lancet. 2014;384(9940):319-328.

49. Schlumberger M, Tahara M, Wirth LJ, Robinson B, Brose MS, Elisei R, Habra MA, Newbold K, Shah MH, Hoff AO, Gianoukakis AG, Kiyota N, Taylor MH, Kim SB, Krzyzanowska MK, Dutcus CE, de las Heras B, Zhu J, Sherman SI. Lenvatinib versus placebo in radioiodine-refractory thyroid cancer. N Engl J Med. 2015;372(7):621-630.

50. Eisen T, Ahmad T, Flaherty KT, Gore M, Kaye S, Marais R, Gibbens I, Hackett S, James M, Schuchter LM, Nathanson KL, Xia C, Simantov R, Schwartz B, Poulin-Costello M, O'Dwyer PJ, Ratain MJ. Sorafenib in advanced melanoma: a Phase II randomised discontinuation trial analysis. Br J Cancer. 2006;95(5):581-586.

51. Falchook GS, Millward M, Hong D, Naing A, Piha-Paul S, Waguespack SG, Cabanillas ME, Sherman SI, Ma B, Curtis M, Goodman V, Kurzrock R. BRAF inhibitor dabrafenib in patients with metastatic BRAF-mutant thyroid cancer. Thyroid. 2015;25(1):71-77.

52. Dunn LA, Sherman EJ, Baxi SS, Tchekmedyian V, Grewal RK, Larson SM, Pentlow KS, Haque S, Tuttle RM, Sabra MM, Fish S, Boucai L, Walters J, Ghossein RA, Seshan VE, Ni A, Li D, Knauf JA, Pfister DG, Fagin JA, Ho AL. Vemurafenib Redifferentiation of BRAF Mutant, RAI-Refractory Thyroid Cancers. J Clin Endocrinol Metab. 2019;104(5):1417-1428.

53. Zhao Y, Adjei AA. The clinical development of MEK inhibitors. Nat Rev Clin Oncol. 2014;11(7):385-400.

54. Croce L, Coperchini F, Magri F, Chiovato L, Rotondi M. The multifaceted anti-cancer effects of BRAF-inhibitors. Oncotarget. 2019;10(61):6623-6640.

55. Drilon A, Laetsch TW, Kummar S, DuBois SG, Lassen UN, Demetri GD, Nathenson M, Doebele RC, Farago AF, Pappo AS, Turpin B, Dowlati A, Brose MS, Mascarenhas L, Federman N, Berlin J, El-Deiry WS, Baik C, Deeken J, Boni V, Nagasubramanian R, Taylor M, Rudzinski ER, Meric-Bernstam F, Sohal DPS, Ma PC, Raez LE, Hechtman JF, Benayed R, Ladanyi M, Tuch BB, Ebata K, Cruickshank S, Ku NC, Cox MC, Hawkins DS, Hong DS, Hyman DM. Efficacy of Larotrectinib in TRK Fusion-Positive Cancers in Adults and Children. N Engl J Med. 2018;378(8):731-739.

56. French JD, Bible K, Spitzweg C, Haugen BR, Ryder M. Leveraging the immune system to treat advanced thyroid cancers. Lancet Diabetes Endocrinol. 2017;5(6):469-481.

57. Cho YY, Kang MJ, Kim SK, Jung JH, Hahm JR, Kim TH, Nam JY, Lee BW, Lee YH, Chung JH, Song SO, Kim SW. Protective Effect of Metformin Against Thyroid Cancer Development: A Population-Based Study in Korea. Thyroid. 2018;28(7):864-870.

58. Shin HS, Sun HJ, Whang YM, Park YJ, Park DJ, Cho SW. Metformin Reduces Thyroid Cancer Tumor Growth in the Metastatic Niche of Bone by Inhibiting Osteoblastic RANKL Productions. Thyroid. 2021;31(5):760-771.

59. Foote RL, Molina JR, Kasperbauer JL, Lloyd RV, McIver B, Morris JC, Grant CS, Thompson GB, Richards ML, Hay ID, Smallridge RC, Bible KC. Enhanced survival in locoregionally confined anaplastic thyroid carcinoma: a single-institution experience using aggressive multimodal therapy. Thyroid. 2011;21(1):25-30.

60. Hsu DS, Kim MK, Balakumaran BS, Acharya CR, Anders CK, Clay T, Lyerly HK, Drake CG, Morse MA, Febbo PG. Immune signatures predict prognosis in localized cancer. Cancer Invest. 2010;28(7):765-773.

61. Chew V, Chen J, Lee D, Loh E, Lee J, Lim KH, Weber A, Slankamenac K, Poon RT, Yang H, Ooi LL, Toh HC, Heikenwalder M, Ng IO, Nardin A, Abastado JP. Chemokine-driven lymphocyte infiltration: an early intratumoural event determining long-term survival in resectable hepatocellular carcinoma. Gut. 2012;61(3):427-438.

62. Gasser S, Lim LHK, Cheung FSG. The role of the tumour microenvironment in immunotherapy. Endocr Relat Cancer. 2017;24(12):T283-T295.

63. Whiteside TL. The tumor microenvironment and its role in promoting tumor growth. Oncogene. 2008;27(45):5904-5912.

64. Raman D, Baugher PJ, Thu YM, Richmond A. Role of chemokines in tumor growth. Cancer Lett. 2007;256(2):137-165.

65. Coperchini F, Croce L, Marinò M, Chiovato L, Rotondi M. Role of chemokine receptors in thyroid cancer and immunotherapy. Endocr Relat Cancer. 2019.

66. Zlotnik A, Burkhardt AM, Homey B. Homeostatic chemokine receptors and organ-specific metastasis. Nat Rev Immunol. 2011;11(9):597-606.

67. Bacon K, Baggiolini M, Broxmeyer H, Horuk R, Lindley I, Mantovani A, Maysushima K, Murphy P, Nomiyama H, Oppenheim J, Rot A, Schall T, Tsang M, Thorpe R, Van Damme J, Wadhwa M, Yoshie O, Zlotnik A, Zoon K, Nomenclature IWSoC. Chemokine/chemokine receptor nomenclature. J Interferon Cytokine Res. 2002;22(10):1067-1068.

68. Legler DF, Thelen M. New insights in chemokine signaling. F1000Res. 2018;7:95.

69. Balkwill FR. The chemokine system and cancer. J Pathol. 2012;226(2):148-157.

70. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature. 2008;454(7203):436-444.

71. Raman D, Sobolik-Delmaire T, Richmond A. Chemokines in health and disease. Exp Cell Res. 2011;317(5):575-589.

72. Cunha LL, Marcello MA, Ward LS. The role of the inflammatory microenvironment in thyroid carcinogenesis. Endocr Relat Cancer. 2014;21(3):R85-R103.

73. Yapa S, Mulla O, Green V, England J, Greenman J. The Role of Chemokines in Thyroid Carcinoma. Thyroid. 2017;27(11):1347-1359.

74. Rotondi M, Chiovato L, Romagnani S, Serio M, Romagnani P. Role of chemokines in endocrine autoimmune diseases. Endocr Rev. 2007;28(5):492-520.

75. Antonelli A, Ferrari SM, Giuggioli D, Ferrannini E, Ferri C, Fallahi P. Chemokine (C-X-C motif) ligand (CXCL)10 in autoimmune diseases. Autoimmun Rev. 2014;13(3):272-280.

76. Melillo RM, Castellone MD, Guarino V, De Falco V, Cirafici AM, Salvatore G, Caiazzo F, Basolo F, Giannini R, Kruhoffer M, Orntoft T, Fusco A, Santoro M. The RET/PTC-RAS-BRAF linear signaling cascade mediates the motile and mitogenic phenotype of thyroid cancer cells. J Clin Invest. 2005;115(4):1068-1081.

77. Xing M. BRAF mutation in papillary thyroid cancer: pathogenic role, molecular bases, and clinical implications. Endocr Rev. 2007;28(7):742-762.

78. Castellone MD, Guarino V, De Falco V, Carlomagno F, Basolo F, Faviana P, Kruhoffer M, Orntoft T, Russell JP, Rothstein JL, Fusco A, Santoro M, Melillo RM. Functional expression of the CXCR4 chemokine receptor is induced by RET/PTC oncogenes and is a common event in human papillary thyroid carcinomas. Oncogene. 2004;23(35):5958-5967.

79. Torregrossa L, Faviana P, Filice ME, Materazzi G, Miccoli P, Vitti P, Fontanini G, Melillo RM, Santoro M, Basolo F. CXC chemokine receptor 4 immunodetection in the follicular variant of papillary thyroid carcinoma: comparison to galectin-3 and hector battifora mesothelial cell-1. Thyroid. 2010;20(5):495-504.

80. Coperchini F, Pignatti P, Leporati P, Carbone A, Croce L, Magri F, Chiovato L, Rotondi M. Normal human thyroid cells, BCPAP, and TPC-1 thyroid tumor cell lines display different profile in both basal and TNF-α-induced CXCL8 secretion. Endocrine. 2016;54(1):123-128.

81. Rotondi M, Coperchini F, Latrofa F, Chiovato L. Role of Chemokines in Thyroid Cancer Microenvironment: Is CXCL8 the Main Player? Front Endocrinol (Lausanne). 2018;9:314.

82. Xie K. Interleukin-8 and human cancer biology. Cytokine Growth Factor Rev. 2001;12(4):375-391.

83. Walz A, Peveri P, Aschauer H, Baggiolini M. Purification and amino acid sequencing of NAF, a novel neutrophil-activating factor produced by monocytes. Biochem Biophys Res Commun. 1987;149(2):755-761.

84. Yoshimura T, Matsushima K, Tanaka S, Robinson EA, Appella E, Oppenheim JJ, Leonard EJ. Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines. Proc Natl Acad Sci U S A. 1987;84(24):9233-9237.

85. Baggiolini M, Walz A, Kunkel SL. Neutrophil-activating peptide-1/interleukin 8, a novel cytokine that activates neutrophils. J Clin Invest. 1989;84(4):1045-1049.

86. De Larco JE, Wuertz BR, Furcht LT. The potential role of neutrophils in promoting the metastatic phenotype of tumors releasing interleukin-8. Clin Cancer Res. 2004;10(15):4895-4900.

87. Waugh DJ, Wilson C. The interleukin-8 pathway in cancer. Clin Cancer Res. 2008;14(21):6735-6741.

88. Roebuck KA. Regulation of interleukin-8 gene expression. J Interferon Cytokine Res. 1999;19(5):429-438.

89. Zachariae CO, Thestrup-Pedersen K, Matsushima K. Expression and secretion of leukocyte chemotactic cytokines by normal human melanocytes and melanoma cells. J Invest Dermatol. 1991;97(3):593-599.

90. Kaskas NM, Moore-Medlin T, McClure GB, Ekshyyan O, Vanchiere JA, Nathan CA. Serum biomarkers in head and neck squamous cell cancer. JAMA Otolaryngol Head Neck Surg. 2014;140(1):5-11.

91. Tjiong MY, van der Vange N, ten Kate FJ, Tjong-A-Hung SP, ter Schegget J, Burger MP, Out TA. Increased IL-6 and IL-8 levels in cervicovaginal secretions of patients with cervical cancer. Gynecol Oncol. 1999;73(2):285-291.

92. Ivarsson K, Runesson E, Sundfeldt K, Haeger M, Hedin L, Janson PO, Brännström M. The chemotactic cytokine interleukin-8--a cyst fluid marker for malignant epithelial ovarian cancer? Gynecol Oncol. 1998;71(3):420-423.

93. Orditura M, De Vita F, Catalano G, Infusino S, Lieto E, Martinelli E, Morgillo F, Castellano P, Pignatelli C, Galizia G. Elevated serum levels of interleukin-8 in advanced non-small cell lung cancer patients: relationship with prognosis. J Interferon Cytokine Res. 2002;22(11):1129-1135.

94. Brew R, Erikson JS, West DC, Kinsella AR, Slavin J, Christmas SE. Interleukin-8 as an autocrine growth factor for human colon carcinoma cells in vitro. Cytokine. 2000;12(1):78-85.

95. Kitadai Y, Haruma K, Sumii K, Yamamoto S, Ue T, Yokozaki H, Yasui W, Ohmoto Y, Kajiyama G, Fidler IJ, Tahara E. Expression of interleukin-8 correlates with vascularity in human gastric carcinomas. Am J Pathol. 1998;152(1):93-100.

96. Venkatakrishnan G, Salgia R, Groopman JE. Chemokine receptors CXCR-1/2 activate mitogen-activated protein kinase via the epidermal growth factor receptor in ovarian cancer cells. J Biol Chem. 2000;275(10):6868-6875.

97. Takamori H, Oades ZG, Hoch OC, Burger M, Schraufstatter IU. Autocrine growth effect of IL-8 and GROalpha on a human pancreatic cancer cell line, Capan-1. Pancreas. 2000;21(1):52-56.

98. Luppi F, Longo AM, de Boer WI, Rabe KF, Hiemstra PS. Interleukin-8 stimulates cell proliferation in non-small cell lung cancer through epidermal growth factor receptor transactivation. Lung Cancer. 2007;56(1):25-33.

99. Kamohara H, Takahashi M, Ishiko T, Ogawa M, Baba H. Induction of interleukin-8 (CXCL-8) by tumor necrosis factor-alpha and leukemia inhibitory factor in pancreatic carcinoma cells: Impact of CXCL-8 as an autocrine growth factor. Int J Oncol. 2007;31(3):627-632.

100. Heidemann J, Ogawa H, Dwinell MB, Rafiee P, Maaser C, Gockel HR, Otterson MF, Ota DM, Lugering N, Domschke W, Binion DG. Angiogenic effects of interleukin 8 (CXCL8) in human intestinal microvascular endothelial cells are mediated by CXCR2. J Biol Chem. 2003;278(10):8508-8515.

101. Belperio JA, Keane MP, Arenberg DA, Addison CL, Ehlert JE, Burdick MD, Strieter RM. CXC chemokines in angiogenesis. J Leukoc Biol. 2000;68(1):1-8.

102. Azenshtein E, Meshel T, Shina S, Barak N, Keydar I, Ben-Baruch A. The angiogenic factors CXCL8 and VEGF in breast cancer: regulation by an array of pro-malignancy factors. Cancer Lett. 2005;217(1):73-86.

103. Inoue K, Slaton JW, Kim SJ, Perrotte P, Eve BY, Bar-Eli M, Radinsky R, Dinney CP. Interleukin 8 expression regulates tumorigenicity and metastasis in human bladder cancer. Cancer Res. 2000;60(8):2290-2299.

104. Karashima T, Sweeney P, Kamat A, Huang S, Kim SJ, Bar-Eli M, McConkey DJ, Dinney CP. Nuclear factor-kappaB mediates angiogenesis and metastasis of human bladder cancer through the regulation of interleukin-8. Clin Cancer Res. 2003;9(7):2786-2797.

105. Schalper KA, Carleton M, Zhou M, Chen T, Feng Y, Huang SP, Walsh AM, Baxi V, Pandya D, Baradet T, Locke D, Wu Q, Reilly TP, Phillips P, Nagineni V, Gianino N, Gu J, Zhao H, Perez-Gracia JL, Sanmamed MF, Melero I. Elevated serum interleukin-8 is associated with enhanced intratumor neutrophils and reduced clinical benefit of immune-checkpoint inhibitors. Nat Med. 2020;26(5):688-692.

106. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin Invest. 2009;119(6):1420-1428.

107. Cheng XS, Li YF, Tan J, Sun B, Xiao YC, Fang XB, Zhang XF, Li Q, Dong JH, Li M, Qian HH, Yin ZF, Yang ZB. CCL20 and CXCL8 synergize to promote progression and poor survival

outcome in patients with colorectal cancer by collaborative induction of the epithelial-mesenchymal transition. Cancer Lett. 2014;348(1-2):77-87.

108. Suarez-Carmona M, Bourcy M, Lesage J, Leroi N, Syne L, Blacher S, Hubert P, Erpicum C, Foidart JM, Delvenne P, Birembaut P, Noël A, Polette M, Gilles C. Soluble factors regulated by epithelial-mesenchymal transition mediate tumour angiogenesis and myeloid cell recruitment. J Pathol. 2015;236(4):491-504.

109. Sanmamed MF, Carranza-Rua O, Alfaro C, Oñate C, Martín-Algarra S, Perez G, Landazuri SF, Gonzalez A, Gross S, Rodriguez I, Muñoz-Calleja C, Rodríguez-Ruiz M, Sangro B, López-Picazo JM, Rizzo M, Mazzolini G, Pascual JI, Andueza MP, Perez-Gracia JL, Melero I. Serum interleukin-8 reflects tumor burden and treatment response across malignancies of multiple tissue origins. Clin Cancer Res. 2014;20(22):5697-5707.

110. Sanmamed MF, Perez-Gracia JL, Schalper KA, Fusco JP, Gonzalez A, Rodriguez-Ruiz ME, Oñate C, Perez G, Alfaro C, Martín-Algarra S, Andueza MP, Gurpide A, Morgado M, Wang J, Bacchiocchi A, Halaban R, Kluger H, Chen L, Sznol M, Melero I. Changes in serum interleukin-8 (IL-8) levels reflect and predict response to anti-PD-1 treatment in melanoma and non-small-cell lung cancer patients. Ann Oncol. 2017;28(8):1988-1995.

111. Weetman AP, Bennett GL, Wong WL. Thyroid follicular cells produce interleukin-8. J Clin Endocrinol Metab. 1992;75(1):328-330.

112. Yamazaki K, Tanigawa K, Suzuki K, Yamada E, Yamada T, Takano K, Obara T, Sato K. Iodide-induced chemokines and genes related to immunological function in cultured human thyroid follicles in the presence of thyrotropin. Thyroid. 2010;20(1):67-76.

113. Corton JM, Gillespie JG, Hawley SA, Hardie DG. 5-aminoimidazole-4-carboxamide ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells? Eur J Biochem. 1995;229(2):558-565.

114. Yoshida M, Matsuzaki H, Sakata K, Takeya M, Kato K, Mizushima S, Kawakita M, Takatsuki K. Neutrophil chemotactic factors produced by a cell line from thyroid carcinoma. Cancer Res. 1992;52(2):464-469.

115. Basolo F, Giannini R, Toniolo A, Casalone R, Nikiforova M, Pacini F, Elisei R, Miccoli P, Berti P, Faviana P, Fiore L, Monaco C, Pierantoni GM, Fedele M, Nikiforov YE, Santoro M, Fusco A. Establishment of a non-tumorigenic papillary thyroid cell line (FB-2) carrying the RET/PTC1 rearrangement. Int J Cancer. 2002;97(5):608-614.

116. Rotondi M, Coperchini F, Awwad O, Pignatti P, Di Buduo CA, Abbonante V, Magri F, Balduini A, Chiovato L. Effect of Interferon-γ on the Basal and the TNFα-Stimulated Secretion of CXCL8 in Thyroid Cancer Cell Lines Bearing Either the RET/PTC Rearrangement Or the BRAF V600e Mutation. Mediators Inflamm. 2016;2016:8512417.

117. Visciano C, Liotti F, Prevete N, Cali' G, Franco R, Collina F, de Paulis A, Marone G, Santoro M, Melillo RM. Mast cells induce epithelial-to-mesenchymal transition and stem cell features in human thyroid cancer cells through an IL-8-Akt-Slug pathway. Oncogene. 2015;34(40):5175-5186.

118. Fang W, Ye L, Shen L, Cai J, Huang F, Wei Q, Fei X, Chen X, Guan H, Wang W, Li X, Ning G. Tumor-associated macrophages promote the metastatic potential of thyroid papillary cancer by releasing CXCL8. Carcinogenesis. 2014;35(8):1780-1787.

119. Nagarsheth N, Wicha MS, Zou W. Chemokines in the cancer microenvironment and their relevance in cancer immunotherapy. Nat Rev Immunol. 2017;17(9):559-572.

120. Allavena P, Germano G, Marchesi F, Mantovani A. Chemokines in cancer related inflammation. Exp Cell Res. 2011;317(5):664-673.

121. Proudfoot AE, Power CA, Rommel C, Wells TN. Strategies for chemokine antagonists as therapeutics. Semin Immunol. 2003;15(1):57-65.

122. Johnson Z, Schwarz M, Power CA, Wells TN, Proudfoot AE. Multi-faceted strategies to combat disease by interference with the chemokine system. Trends Immunol. 2005;26(5):268-274.

123. Bonapace L, Coissieux MM, Wyckoff J, Mertz KD, Varga Z, Junt T, Bentires-Alj M. Cessation of CCL2 inhibition accelerates breast cancer metastasis by promoting angiogenesis. Nature. 2014;515(7525):130-133.

124. Long H, Xie R, Xiang T, Zhao Z, Lin S, Liang Z, Chen Z, Zhu B. Autocrine CCL5 signaling promotes invasion and migration of CD133+ ovarian cancer stem-like cells via NF-κB-mediated MMP-9 upregulation. Stem Cells. 2012;30(10):2309-2319.

125. Ha H, Debnath B, Neamati N. Role of the CXCL8-CXCR1/2 Axis in Cancer and Inflammatory Diseases. Theranostics. 2017;7(6):1543-1588.

126. Liotti F, De Pizzol M, Allegretti M, Prevete N, Melillo RM. Multiple anti-tumor effects of Reparixin on thyroid cancer. Oncotarget. 2017;8(22):35946-35961.

127. Rotondi M, Coperchini F, Sideri R, Groppelli G, de Martinis L, Villani L, Pignatti P, Magri F, Chiovato L. Type I and type II interferons inhibit both basal and tumor necrosis factor-α-induced CXCL8 secretion in primary cultures of human thyrocytes. J Interferon Cytokine Res. 2013;33(9):508-513.

128. Rotondi M, Coperchini F, Pignatti P, Magri F, Chiovato L. Metformin reverts the secretion of CXCL8 induced by TNF-α in primary cultures of human thyroid cells: an additional indirect anti-tumor effect of the drug. J Clin Endocrinol Metab. 2015;100(3):E427-432.

129. Awwad O, Coperchini F, Pignatti P, Denegri M, Massara S, Croce L, Di Buduo CA, Abbonante V, Balduini A, Chiovato L, Rotondi M. The AMPK-activator AICAR in thyroid cancer: effects on CXCL8 secretion and on CXCL8-induced neoplastic cell migration. J Endocrinol Invest. 2018.

130. Jung YH, Lee DY, Cha W, Kim BH, Sung MW, Kim KH, Ahn SH. Antitumor effect of CXCR4 antagonist AMD3100 on the tumorigenic cell line of BHP10-3 papillary thyroid cancer cells. Head Neck. 2016;38(10):1479-1486.

131. Caturegli P, De Remigis A, Chuang K, Dembele M, Iwama A, Iwama S. Hashimoto's thyroiditis: celebrating the centennial through the lens of the Johns Hopkins hospital surgical pathology records. Thyroid. 2013;23(2):142-150.

132. Caturegli P, De Remigis A, Rose NR. Hashimoto thyroiditis: clinical and diagnostic criteria. Autoimmun Rev. 2014;13(4-5):391-397.

133. Croce L, De Martinis L, Pinto S, Coperchini F, Dito G, Bendotti G, Pasquali D, Cappelli C, Latrofa F, Magri F, Chiovato L, Rotondi M. Compared with classic Hashimoto's thyroiditis, chronic autoimmune serum-negative thyroiditis requires a lower substitution dose of L-thyroxine to correct hypothyroidism. J Endocrinol Invest. 2020.

134. Ragusa F, Fallahi P, Elia G, Gonnella D, Paparo SR, Giusti C, Churilov LP, Ferrari SM, Antonelli A. Hashimotos' thyroiditis: Epidemiology, pathogenesis, clinic and therapy. Best Pract Res Clin Endocrinol Metab. 2019;33(6):101367.

135. Ehlers M, Thiel A, Bernecker C, Porwol D, Papewalis C, Willenberg HS, Schinner S, Hautzel H, Scherbaum WA, Schott M. Evidence of a combined cytotoxic thyroglobulin and thyroperoxidase epitope-specific cellular immunity in Hashimoto's thyroiditis. J Clin Endocrinol Metab. 2012;97(4):1347-1354.

136. Liu M, Zhao L, Gao Y, Huang Y, Lu G, Guo X. Epitope recognition patterns of thyroglobulin antibody in sera from patients with Hashimoto's thyroiditis on different thyroid functional status. Clin Exp Immunol. 2012;170(3):283-290.

137. Nielsen CH, Brix TH, Gardas A, Banga JP, Hegedüs L. Epitope recognition patterns of thyroid peroxidase autoantibodies in healthy individuals and patients with Hashimoto's thyroiditis*. Clin Endocrinol (Oxf). 2008;69(4):664-668.

138. Quaratino S, Badami E, Pang YY, Bartok I, Dyson J, Kioussis D, Londei M, Maiuri L. Degenerate self-reactive human T-cell receptor causes spontaneous autoimmune disease in mice. Nat Med. 2004;10(9):920-926.

139. Watanabe H, Inaba M, Adachi Y, Sugiura K, Hisha H, Iguchi T, Ito T, Yasumizu R, Inaba K, Yamashita T, Ikehara S. Experimental autoimmune thyroiditis induced by thyroglobulin-pulsed dendritic cells. Autoimmunity. 1999;31(4):273-282.

140. Kabel PJ, Voorbij HA, De Haan M, van der Gaag RD, Drexhage HA. Intrathyroidal dendritic cells. J Clin Endocrinol Metab. 1988;66(1):199-207.

141. Glick AB, Wodzinski A, Fu P, Levine AD, Wald DN. Impairment of regulatory T-cell function in autoimmune thyroid disease. Thyroid. 2013;23(7):871-878.

142. Domberg J, Liu C, Chao L, Papewalis C, Pfleger C, Xu K, Willenberg HS, Hermsen D, Scherbaum WA, Schloot NC, Schott M. Circulating chemokines in patients with autoimmune thyroid diseases. Horm Metab Res. 2008;40(6):416-421.

143. Vural P, Degirmencioglu S, Erden S, Gelincik A. The relationship between transforming growth factor-beta1, vascular endothelial growth factor, nitric oxide and Hashimoto's thyroiditis. Int Immunopharmacol. 2009;9(2):212-215.

144. Rotondi M, Lazzeri E, Romagnani P, Serio M. Role for interferon-gamma inducible chemokines in endocrine autoimmunity: an expanding field. J Endocrinol Invest. 2003;26(2):177-180.

145. Rotondi M, Coperchini F, Pignatti P, Sideri R, Groppelli G, Leporati P, La Manna L, Magri F, Mariotti S, Chiovato L. Interferon-γ and tumor necrosis factor-α sustain secretion of specific CXC chemokines in human thyrocytes: a first step toward a differentiation between autoimmune and tumor-related inflammation? J Clin Endocrinol Metab. 2013;98(1):308-313.

146. Surks MI, Hollowell JG. Age-specific distribution of serum thyrotropin and antithyroid antibodies in the US population: implications for the prevalence of subclinical hypothyroidism. J Clin Endocrinol Metab. 2007;92(12):4575-4582.

147. Vanderpump MP, Tunbridge WM, French JM, Appleton D, Bates D, Clark F, Grimley Evans J, Hasan DM, Rodgers H, Tunbridge F. The incidence of thyroid disorders in the community: a twenty-year follow-up of the Whickham Survey. Clin Endocrinol (Oxf). 1995;43(1):55-68.

148. Rotondi M, de Martinis L, Coperchini F, Pignatti P, Pirali B, Ghilotti S, Fonte R, Magri F, Chiovato L. Serum negative autoimmune thyroiditis displays a milder clinical picture compared with classic Hashimoto's thyroiditis. Eur J Endocrinol. 2014;171(1):31-36.

149. DAILEY ME, LINDSAY S, SKAHEN R. Relation of thyroid neoplasms to Hashimoto disease of the thyroid gland. AMA Arch Surg. 1955;70(2):291-297.

150. Liotti F, Visciano C, Melillo RM. Inflammation in thyroid oncogenesis. Am J Cancer Res. 2012;2(3):286-297.

151. Guarino V, Castellone MD, Avilla E, Melillo RM. Thyroid cancer and inflammation. Mol Cell Endocrinol. 2010;321(1):94-102.

152. French JD, Weber ZJ, Fretwell DL, Said S, Klopper JP, Haugen BR. Tumor-associated lymphocytes and increased FoxP3+ regulatory T cell frequency correlate with more aggressive papillary thyroid cancer. J Clin Endocrinol Metab. 2010;95(5):2325-2333.

153. Slijepcevic N, Zivaljevic V, Marinkovic J, Sipetic S, Diklic A, Paunovic I. Retrospective evaluation of the incidental finding of 403 papillary thyroid microcarcinomas in 2466 patients undergoing thyroid surgery for presumed benign thyroid disease. BMC Cancer. 2015;15:330.

154. Azizi G, Keller JM, Lewis M, Piper K, Puett D, Rivenbark KM, Malchoff CD. Association of Hashimoto's thyroiditis with thyroid cancer. Endocr Relat Cancer. 2014;21(6):845-852.

155. Boi F, Minerba L, Lai ML, Marziani B, Figus B, Spanu F, Borghero A, Mariotti S. Both thyroid autoimmunity and increased serum TSH are independent risk factors for malignancy in patients with thyroid nodules. J Endocrinol Invest. 2013;36(5):313-320.

156. Kim ES, Lim DJ, Baek KH, Lee JM, Kim MK, Kwon HS, Song KH, Kang MI, Cha BY, Lee KW, Son HY. Thyroglobulin antibody is associated with increased cancer risk in thyroid nodules. Thyroid. 2010;20(8):885-891.

157. Iliadou PK, Effraimidis G, Konstantinos M, Grigorios P, Mitsakis P, Patakiouta F, Pazaitou-Panayiotou K. Chronic lymphocytic thyroiditis is associated with invasive characteristics of differentiated thyroid carcinoma in children and adolescents. Eur J Endocrinol. 2015;173(6):827-833.

158. Paparodis R, Imam S, Todorova-Koteva K, Staii A, Jaume JC. Hashimoto's thyroiditis pathology and risk for thyroid cancer. Thyroid. 2014;24(7):1107-1114.

159. Repplinger D, Bargren A, Zhang YW, Adler JT, Haymart M, Chen H. Is Hashimoto's thyroiditis a risk factor for papillary thyroid cancer? J Surg Res. 2008;150(1):49-52.

160. Mukasa K, Noh JY, Kunii Y, Matsumoto M, Sato S, Yasuda S, Suzuki M, Ito K. Prevalence of malignant tumors and adenomatous lesions detected by ultrasonographic screening in patients with autoimmune thyroid diseases. Thyroid. 2011;21(1):37-41.

161. Zhang Y, Dai J, Wu T, Yang N, Yin Z. The study of the coexistence of Hashimoto's thyroiditis with papillary thyroid carcinoma. J Cancer Res Clin Oncol. 2014;140(6):1021-1026.

162. Dvorkin S, Robenshtok E, Hirsch D, Strenov Y, Shimon I, Benbassat CA. Differentiated thyroid cancer is associated with less aggressive disease and better outcome in patients with coexisting Hashimotos thyroiditis. J Clin Endocrinol Metab. 2013;98(6):2409-2414.

163. Baser H, Ozdemir D, Cuhaci N, Aydin C, Ersoy R, Kilicarslan A, Cakir B. Hashimoto's Thyroiditis Does Not Affect Ultrasonographical, Cytological, and Histopathological Features in Patients with Papillary Thyroid Carcinoma. Endocr Pathol. 2015;26(4):356-364.

164. Castagna MG, Belardini V, Memmo S, Maino F, Di Santo A, Toti P, Carli AF, Caruso G, Pacini F. Nodules in autoimmune thyroiditis are associated with increased risk of thyroid cancer in surgical series but not in cytological series: evidence for selection bias. J Clin Endocrinol Metab. 2014;99(9):3193-3198.

165. Donangelo I, Walts AE, Bresee C, Braunstein GD. LYMPHOCYTIC THYROIDITIS IS ASSOCIATED WITH INCREASED NUMBER OF BENIGN CERVICAL NODES AND FEWER CENTRAL NECK COMPARTMENT METASTATIC LYMPH NODES IN PATIENTS WITH DIFFERENTIATED THYROID CANCER. Endocr Pract. 2016;22(10):1192-1198.

166. Giagourta I, Evangelopoulou C, Papaioannou G, Kassi G, Zapanti E, Prokopiou M, Papapostolou K, Karga H. Autoimmune thyroiditis in benign and malignant thyroid nodules: 16-year results. Head Neck. 2014;36(4):531-535.

167. Kim SK, Woo JW, Lee JH, Park I, Choe JH, Kim JH, Kim JS. Chronic lymphocytic thyroiditis and BRAF V600E in papillary thyroid carcinoma. Endocr Relat Cancer. 2016;23(1):27-34.

168. Qu N, Zhang L, Lin DZ, Ji QH, Zhu YX, Wang Y. The impact of coexistent Hashimoto's thyroiditis on lymph node metastasis and prognosis in papillary thyroid microcarcinoma. Tumour Biol. 2016;37(6):7685-7692.

169. Radetti G, Loche S, D'Antonio V, Salerno M, Guzzetti C, Aversa T, Cassio A, Cappa M, Gastaldi R, Deluca F, Vigone MC, Tronconi GM, Corrias A. Influence of Hashimoto Thyroiditis on

the Development of Thyroid Nodules and Cancer in Children and Adolescents. J Endocr Soc. 2019;3(3):607-616.

170. Jankovic B, Le KT, Hershman JM. Clinical Review: Hashimoto's thyroiditis and papillary thyroid carcinoma: is there a correlation? J Clin Endocrinol Metab. 2013;98(2):474-482.

171. Kurukahvecioglu O, Taneri F, Yüksel O, Aydin A, Tezel E, Onuk E. Total thyroidectomy for the treatment of Hashimoto's thyroiditis coexisting with papillary thyroid carcinoma. Adv Ther. 2007;24(3):510-516.

172. Haymart MR, Banerjee M, Reyes-Gastelum D, Caoili E, Norton EC. Thyroid Ultrasound and the Increase in Diagnosis of Low-Risk Thyroid Cancer. J Clin Endocrinol Metab. 2019;104(3):785-792.

173. Jackson D, Handelsman RS, Farrá JC, Lew JI. Increased Incidental Thyroid Cancer in Patients With Subclinical Chronic Lymphocytic Thyroiditis. J Surg Res. 2020;245:115-118.

174. Holm LE, Blomgren H, Löwhagen T. Cancer risks in patients with chronic lymphocytic thyroiditis. N Engl J Med. 1985;312(10):601-604.

175. Singh B, Shaha AR, Trivedi H, Carew JF, Poluri A, Shah JP. Coexistent Hashimoto's thyroiditis with papillary thyroid carcinoma: impact on presentation, management, and outcome. Surgery. 1999;126(6):1070-1076; discussion 1076-1077.

176. Yoon YH, Kim HJ, Lee JW, Kim JM, Koo BS. The clinicopathologic differences in papillary thyroid carcinoma with or without co-existing chronic lymphocytic thyroiditis. Eur Arch Otorhinolaryngol. 2012;269(3):1013-1017.

177. Moon S, Chung HS, Yu JM, Yoo HJ, Park JH, Kim DS, Park YJ. Associations between Hashimoto Thyroiditis and Clinical Outcomes of Papillary Thyroid Cancer: A Meta-Analysis of Observational Studies. Endocrinol Metab (Seoul). 2018;33(4):473-484.

178. Kim EY, Kim WG, Kim WB, Kim TY, Kim JM, Ryu JS, Hong SJ, Gong G, Shong YK. Coexistence of chronic lymphocytic thyroiditis is associated with lower recurrence rates in patients with papillary thyroid carcinoma. Clin Endocrinol (Oxf). 2009;71(4):581-586.

179. Kim KH, Suh KS, Kang DW, Kang DY. Mutations of the BRAF gene in papillary thyroid carcinoma and in Hashimoto's thyroiditis. Pathol Int. 2005;55(9):540-545.

180. Cunha LL, Morari EC, Guihen AC, Razolli D, Gerhard R, Nonogaki S, Soares FA, Vassallo J, Ward LS. Infiltration of a mixture of immune cells may be related to good prognosis in patients with differentiated thyroid carcinoma. Clin Endocrinol (Oxf). 2012;77(6):918-925.

181. French JD, Kotnis GR, Said S, Raeburn CD, McIntyre RC, Klopper JP, Haugen BR. Programmed death-1+ T cells and regulatory T cells are enriched in tumor-involved lymph nodes and associated with aggressive features in papillary thyroid cancer. J Clin Endocrinol Metab. 2012;97(6):E934-943.

182. Qing W, Fang WY, Ye L, Shen LY, Zhang XF, Fei XC, Chen X, Wang WQ, Li XY, Xiao JC, Ning G. Density of tumor-associated macrophages correlates with lymph node metastasis in papillary thyroid carcinoma. Thyroid. 2012;22(9):905-910.

183. Yu H, Huang X, Liu X, Jin H, Zhang G, Zhang Q, Yu J. Regulatory T cells and plasmacytoid dendritic cells contribute to the immune escape of papillary thyroid cancer coexisting with multinodular non-toxic goiter. Endocrine. 2013;44(1):172-181.

184. Pani F, Yasuda Y, Di Dalmazi G, Chalan P, Gabrielson K, Adamo L, Sabini E, Mariotti S, Caturegli P. Pre-existing thyroiditis ameliorates papillary thyroid cancer: insights from a new mouse model. Endocrinology. 2021.

185. Coperchini F, Pignatti P, Carbone A, Bongianino R, Di Buduo CA, Leporati P, Croce L, Magri F, Balduini A, Chiovato L, Rotondi M. TNF-α increases the membrane expression of the chemokine

receptor CCR6 in thyroid tumor cells, but not in normal thyrocytes: potential role in the metastatic spread of thyroid cancer. Tumour Biol. 2016;37(4):5569-5575.

186. Abbonante V, Gruppi C, Rubel D, Gross O, Moratti R, Balduini A. Discoidin domain receptor 1 protein is a novel modulator of megakaryocyte-collagen interactions. J Biol Chem. 2013;288(23):16738-16746.

187. Jackson AL, Sun W, Kilgore J, Guo H, Fang Z, Yin Y, Jones HM, Gilliam TP, Zhou C, Bae-Jump VL. Phenformin has anti-tumorigenic effects in human ovarian cancer cells and in an orthotopic mouse model of serous ovarian cancer. Oncotarget. 2017;8(59):100113-100127.

188. Pistoni M, Tondelli G, Gallo C, Torricelli F, Maresca A, Carelli V, Ciarrocchi A, Dallaglio K. Exploring metabolic reprogramming in melanoma via acquired resistance to the oxidative phosphorylation inhibitor phenformin. Melanoma Res. 2019.

189. Miskimins WK, Ahn HJ, Kim JY, Ryu S, Jung YS, Choi JY. Synergistic anti-cancer effect of phenformin and oxamate. PLoS One. 2014;9(1):e85576.

190. Franken NA, Rodermond HM, Stap J, Haveman J, van Bree C. Clonogenic assay of cells in vitro. Nat Protoc. 2006;1(5):2315-2319.

191. Crowley LC, Christensen ME, Waterhouse NJ. Measuring Survival of Adherent Cells with the Colony-Forming Assay. Cold Spring Harb Protoc. 2016;2016(8).

192. Rotondi M, Coperchini F, Magri F, Chiovato L. Serum-negative autoimmune thyroiditis: what's in a name? J Endocrinol Invest. 2014;37(6):589-591.

193. Russ G, Bonnema SJ, Erdogan MF, Durante C, Ngu R, Leenhardt L. European Thyroid Association Guidelines for Ultrasound Malignancy Risk Stratification of Thyroid Nodules in Adults: The EU-TIRADS. Eur Thyroid J. 2017;6(5):225-237.

194. Rotondi M CF, Latrofa F, Chiovato L. Role of chemokines in thyroid cancermicroenvironment: is CXCL8 the main player? . Vol in Press. Frontiers in Endocrinology2018.

195. Coperchini F, Croce L, Denegri M, Awwad O, Ngnitejeu ST, Muzza M, Capelli V, Latrofa F, Persani L, Chiovato L, Rotondi M. The BRAF-inhibitor PLX4720 inhibits CXCL8 secretion in BRAFV600E mutated and normal thyroid cells: a further anti-cancer effect of BRAF-inhibitors. Sci Rep. 2019;9(1):4390.

196. Tsai J, Lee JT, Wang W, Zhang J, Cho H, Mamo S, Bremer R, Gillette S, Kong J, Haass NK, Sproesser K, Li L, Smalley KS, Fong D, Zhu YL, Marimuthu A, Nguyen H, Lam B, Liu J, Cheung I, Rice J, Suzuki Y, Luu C, Settachatgul C, Shellooe R, Cantwell J, Kim SH, Schlessinger J, Zhang KY, West BL, Powell B, Habets G, Zhang C, Ibrahim PN, Hirth P, Artis DR, Herlyn M, Bollag G. Discovery of a selective inhibitor of oncogenic B-Raf kinase with potent antimelanoma activity. Proc Natl Acad Sci U S A. 2008;105(8):3041-3046.

197. Montero-Conde C, Ruiz-Llorente S, Dominguez JM, Knauf JA, Viale A, Sherman EJ, Ryder M, Ghossein RA, Rosen N, Fagin JA. Relief of feedback inhibition of HER3 transcription by RAF and MEK inhibitors attenuates their antitumor effects in BRAF-mutant thyroid carcinomas. Cancer Discov. 2013;3(5):520-533.

198. Nucera C, Nehs MA, Nagarkatti SS, Sadow PM, Mekel M, Fischer AH, Lin PS, Bollag GE, Lawler J, Hodin RA, Parangi S. Targeting BRAFV600E with PLX4720 displays potent antimigratory and anti-invasive activity in preclinical models of human thyroid cancer. Oncologist. 2011;16(3):296-309.

199. Salerno P, De Falco V, Tamburrino A, Nappi TC, Vecchio G, Schweppe RE, Bollag G, Santoro M, Salvatore G. Cytostatic activity of adenosine triphosphate-competitive kinase inhibitors in BRAF mutant thyroid carcinoma cells. J Clin Endocrinol Metab. 2010;95(1):450-455.

200. Coperchini F, Croce L, Denegri M, Awwad O, Ngnitejeu ST, Magri F, Chiovato L, Rotondi M. The anti-cancer effects of phenformin in thyroid cancer cell lines and in normal thyrocytes. Oncotarget. 2019;10(60):6432-6443.

201. Preto A, Gonçalves J, Rebocho AP, Figueiredo J, Meireles AM, Rocha AS, Vasconcelos HM, Seca H, Seruca R, Soares P, Sobrinho-Simões M. Proliferation and survival molecules implicated in the inhibition of BRAF pathway in thyroid cancer cells harbouring different genetic mutations. BMC Cancer. 2009;9:387.

202. Wang Y, Li W, Phay JE, Shen R, Pellegata NS, Saji M, Ringel MD, de la Chapelle A, He H. Primary Cell Culture Systems for Human Thyroid Studies. Thyroid. 2016;26(8):1131-1140.

203. Coperchini F, Leporati P, Rotondi M, Chiovato L. Expanding the therapeutic spectrum of metformin: from diabetes to cancer. J Endocrinol Invest. 2015;38(10):1047-1055.

204. Rotondi M, Coperchini F, Chiovato L. CXCL8 in thyroid disease: from basic notions to potential applications in clinical practice. Cytokine Growth Factor Rev. 2013;24(6):539-546.

205. Liotti F, Collina F, Pone E, La Sala L, Franco R, Prevete N, Melillo RM. Interleukin-8, but not the Related Chemokine CXCL1, Sustains an Autocrine Circuit Necessary for the Properties and Functions of Thyroid Cancer Stem Cells. Stem Cells. 2017;35(1):135-146.

206. Fernando RI, Castillo MD, Litzinger M, Hamilton DH, Palena C. IL-8 signaling plays a critical role in the epithelial-mesenchymal transition of human carcinoma cells. Cancer Res. 2011;71(15):5296-5306.

207. David JM, Dominguez C, Hamilton DH, Palena C. The IL-8/IL-8R Axis: A Double Agent in Tumor Immune Resistance. Vaccines (Basel). 2016;4(3).

208. Crawford S, Belajic D, Wei J, Riley JP, Dunford PJ, Bembenek S, Fourie A, Edwards JP, Karlsson L, Brunmark A, Wolin RL, Blevitt JM. A novel B-RAF inhibitor blocks interleukin-8 (IL-

8) synthesis in human melanoma xenografts, revealing IL-8 as a potential pharmacodynamic biomarker. Mol Cancer Ther. 2008;7(3):492-499.

209. Fiore E, Latrofa F, Vitti P. Iodine, thyroid autoimmunity and cancer. Eur Thyroid J. 2015;4(1):26-35.

210. Fiore E, Rago T, Provenzale MA, Scutari M, Ugolini C, Basolo F, Di Coscio G, Berti P, Grasso L, Elisei R, Pinchera A, Vitti P. Lower levels of TSH are associated with a lower risk of papillary thyroid cancer in patients with thyroid nodular disease: thyroid autonomy may play a protective role. Endocr Relat Cancer. 2009;16(4):1251-1260.

211. Haymart MR, Repplinger DJ, Leverson GE, Elson DF, Sippel RS, Jaume JC, Chen H. Higher serum thyroid stimulating hormone level in thyroid nodule patients is associated with greater risks of differentiated thyroid cancer and advanced tumor stage. J Clin Endocrinol Metab. 2008;93(3):809-814.

212. Azizi G, Malchoff CD. Autoimmune thyroid disease: a risk factor for thyroid cancer. Endocr Pract. 2011;17(2):201-209.

213. Rotondi M, Groppelli G, Croce L, Latrofa F, Ancona G, Coperchini F, Pasquali D, Cappelli C, Fugazza A, Guazzoni V, Radetti G, Chiovato L. Patients with chronic autoimmune thyroiditis are not at higher risk for developing clinically overt thyroid cancer: a 10-year follow-up study. Eur J Endocrinol. 2020;183(3):317-323.

214. Borowczyk M, Janicki A, Dworacki G, Szczepanek-Parulska E, Danieluk M, Barnett J, Antonik M, Kałużna M, Bromińska B, Czepczyński R, Bączyk M, Ziemnicka K, Ruchała M. Decreased staging of differentiated thyroid cancer in patients with chronic lymphocytic thyroiditis. J Endocrinol Invest. 2019;42(1):45-52.

215. Xu S, Huang H, Qian J, Liu Y, Huang Y, Wang X, Liu S, Xu Z, Liu J. Prevalence of Hashimoto Thyroiditis in Adults With Papillary Thyroid Cancer and Its Association With Cancer Recurrence and Outcomes. JAMA Netw Open. 2021;4(7):e2118526.

216. Bodnar RJ, Yates CC, Rodgers ME, Du X, Wells A. IP-10 induces dissociation of newly formed blood vessels. J Cell Sci. 2009;122(Pt 12):2064-2077.