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Role of type 2 deiodinase in thyroid kinase inhibitors-induced systemic hypothyroidism

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INDEX

Summary

Thyroid cancer accounts for up to 95% of primary cancers involving the endocrine system. Papillary (PTC) and follicular (FTC) thyroid carcinomas arise from thyroid epithelial cells and together make up differentiated thyroid cancer (DTC) (1); Medullary thyroid carcinoma (MTC) arises from the parafollicular cells that produce calcitonin (2).

Thyroid cancer typically has a good outcome following standard treatments, however thyroid cancers that persist or recur following these therapies have a poorer prognosis. 'Target therapy' with tyrosine kinase inhibitors (TKIs) represents an important therapeutic option for the treatment of advanced cases of radioiodine refractory (RAI-R) DTC, MTC and possibly for cases of poorly differentiated (PDTC) and anaplastic thyroid cancer (ATC). In the last few years, several TKIs have been tested for the treatment of advanced, progressive and RAI-R thyroid cancers and some of them have been recently approved for use in clinical practice: sorafenib and lenvatinib for DTC and PDTC; vandetanib and cabozantinib for MTC.

Hypothyroidism is a common side effect during TKIs cancer treatment and occurs in patients that underwent total thyroidectomy for thyroid cancer and in patients treated with TKIs for other cancers and that not underwent thyroidectomy. The clinical relevance of hypothyroidism, the value of thyroid hormone replacement in individuals with abnormal TSH after TKI therapy, and the correct timing of replacement therapy is necessary to improve the therapies used and quality of life for patients with cancer. The etiology of hypothyroidism has not been determined.

We aimed to describe tissutal hypothyroidism especially in the heart and to understand how the TKIs influence the activity of deiodinase. Elucidating the influence of TKIs in the deiodinase activity is crucial for avoid side effects and improve adherence and efficacy of therapy.

1. Introduction

1.1 Thyroid Cancer and TKIs treatment

Thyroid cancer accounts for up to 95% of primary cancers involving the endocrine system. Papillary (PTC) and follicular (FTC) carcinomas, including Hürthle cell carcinoma (HCC), arise from thyroid epithelial cells and together make up differentiated thyroid cancer (DTC) (1). However, epithelial cells also give rise to anaplastic thyroid cancer (ATC), a rare and extremely aggressive type of thyroid cancer that is lethal within a year in the majority of cases. Medullary thyroid carcinoma (MTC) arises from the parafollicular cells that produce calcitonin (2). DTCs, which account for approximately 90% of thyroid cancers, are generally indolent malignancies, in which 80 to 90% of patients survive to 10 years or more. For most DTCs, definitive surgical resection with total thyroidectomy is indicated followed by postoperative radioiodine ablation with ^{131}I (RAI) to destroy residual thyroid tissue.

Thyroid cancer typically has a good outcome following standard treatments, which can include surgery, radioactive iodine ablation for differentiated tumors and treatment with thyrotropin hormone-suppressive levothyroxine. Thyroid cancers that persist or recur following these therapies have a poorer prognosis. Cytotoxic chemotherapy or external beam radiotherapy has a low efficacy in these patients. 'Target therapy' with tyrosine kinase inhibitors (TKIs) represents an important therapeutic option for the treatment of advanced cases of radioiodine refractory (RAI-R) differentiated thyroid cancer (DTC), medullary thyroid cancer (MTC) and possibly for cases of poorly differentiated (PDTC) and anaplastic thyroid cancer (ATC). In the last few years, several TKIs have been tested for the treatment of advanced, progressive and RAI-R thyroid cancers and some of them have been recently approved for use in clinical practice: sorafenib and lenvatinib for DTC and PDTC; vandetanib and cabozantinib for MTC.

According to expert opinion (3), candidates for treatment with TKIs are patients with a RAI-R or MTC metastatic thyroid cancer with lesions radiologically measurable and in progression over the previous 12-14 months, as defined by RECIST (4).

Patients are classified as having RAI-R disease if they have (1):

- lack of RAI uptake on post- therapy scan after RAI-administered activity >30 mCi following appropriate iodine deprivation and adequate thyrotropin (TSH) elevation;

- lack of RAI uptake on a properly conducted diagnostic whole-body scan in the setting of known structural disease, as demonstrated by cross-sectional imaging;

- lack of demonstrable ability of the tumor to concentrate sufficient RAI for tumoricidal effect;

- structural progression of thyroid cancer 6–12 months after RAI therapy;

- a rising thyroglobulin (Tg) level 6–12 months after RAI therapy;

- continued progression of thyroid cancer, despite cumulative RAI-administered activities >500–600 mCi in adult patients.

Due to the presence of two specific serum markers in thyroid cancer that could correlate with the tumor burden, calcitonin (CT) for MTC and Tg for DTC, many researchers have investigated their doubling time to assess the progression of the disease (5, 6). Despite the reliability of these markers, progressive diseases must be assessed with standardized imaging, which should be repeated every 6-12 months, and the rate of progression should be calculated using RECIST.

When the lesions are multiple, distributed in several organs and constantly growing is the right time to consider the possibility of using TKIs that are

active and also safe in old patients, regardless of other concomitant and relevant diseases.

When it is time to start the systemic therapy, it is mandatory to investigate the patient's general health conditions, associations with other diseases, the simultaneous use of other drugs that can interfere with the TKIs or potentiate some adverse events (such as the prolongation of QT). A cardiologist consultation, ECG and a blood pressure measurement are also part of the medical consultation before starting therapy. An accurate pharmacological correction of high blood pressure should be carried out before starting the TKI or during treatment if it increases after starting the TKIs.

Once the drug has been chosen and the treatment has been initiated, the patient must be visited every 15 days in the first 2 months and then less frequently if everything is going well. Major adverse events during treatment with TKIs are reported in the Table 1. At the appearance of adverse events the daily dose of the drug can be reduced or the drug can be stopped according to the lower or greater severity of the adverse event.

Drug	Cancer	Adverse events (all grades) %							
		Hypertension	Diarrhoea	Skin rash	Anorexia	Nausea	Weight loss	Fatigue	QTc prolongation
Vandetanib	MTC	32	56	45	21	33	10	24	14
Cabozantinib	MTC	32	63	19	45	43	47	40	NE
Sorafenib	DTC	40	68	50	32	20	47	50	NE
Lenvatinib	DTC	67	59	15	49	41	45	59	8

Table 1: **Major adverse events reported in the four phase III clinical studies of the TKIs approved for the treatment of advanced thyroid cancer.**

Vandetanib is a small molecule inhibitor of EGFR, VEGFR-2, -3, and RET. Vandetanib is a once-daily oral multitarget TKI that is very active against RET oncogene, VEGF-R and epidermal growth factor receptor (EGF-R). Vandetanib is the first drug approved for the treatment of adult patients with symptomatic, unresectable, locally advanced or metastatic MTC in the USA (2011) and Europe (2013) (Caprelsa, Sanofi-Genzyme). The approval of vandetanib was obtained

after the encouraging results of the international, multicentric and randomised against placebo, ZETA phase III study (7), which showed a significant prolongation of the progression-free survival (PFS) of treated patients with respect to patients treated with placebo (30.5 versus 19.3 months). After the publication of a phase II study specifically dedicated to children affected by the hereditary form of MTC (8), the use of vandetanib has also been approved for children with advanced MTC, usually affected by a hereditary form (9).

The ZETA study showed that the drug is not only able to stop cell growth, as expected and as shown by the prolongation of the progression free survival (PFS), but also induced tumoral shrinkage with a consequent volume reduction of the metastatic lesions.

1.2 Genetic mutations in thyroid cancer

Intracellular signaling pathways involving Ras, B-Raf, and Akt are critical to the molecular pathophysiology of DTC. Ras and Raf are central mediators of the MAP-kinase signaling pathway, and, as such, mediate cell survival and proliferation. Of note, Ras activates both Raf and PI3K as well as many other downstream effector molecules. Though several Raf mutations have been reported in DTC (BRAF T599I-VKSR), the most common (approximately 80-90%) is the BRAF T17699A (V600E) mutation that results in constitutive activation of the kinase, present in 30 to 70% of PTC. Additionally, the multiple isoforms of RAS (H-, K-, and N-RAS) may harbor mutations in DTC (10,11). Estimates of the prevalence of RAS mutations in thyroid tumors vary considerably but are generally reported to be in the range of 25% to 30%. Further studies show that genetic alteration in RAS and RAF in DTC is associated with higher risk phenotypes, more aggressive disease, and poorer overall survival.

More recently, increased activity of the PI3 kinase (PI3K)/Akt pathway has been shown to play a role in the tumorigenesis and progression of DTC. PI3K/Akt signaling promotes tumor cell survival and proliferation. PI3K is activated by

various membrane growth factor receptors such as epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), and platelet-derived growth factor receptor (PDGFR). Activating mutations in PI3K have been identified in ATC cell lines, and increased expression has been seen in PTC tissue. In thyroid cancer cells, PI3K, through its p110 catalytic subunit, activates phos-phatidylinositol-4,5-bisphosphate (PIP2) to phosphatidyl-inositol-3,4,5-trisphosphate (PIP3). PIP3 in turn participates in the phosphorylation of Akt to its active state with a phosphoinositide-dependent kinase, PDK-1. Akt activates the mammalian target of rapamycin (mTOR). The PI3K/Akt pathway is antagonized by the tumor suppressor gene PTEN, whose gene product dephosphorylates PIP3 thus interrupting PI3K signaling. Akt expression also has been shown to lead to the expression of HIF1α, raising the possibility that Akt might play a role in tumor angiogenesis. Akt is overexpressed in sporadic thyroid cancers and induces apoptosis in thyroid cancer cell lines. Point mutations in PIP3 are found in ATC cell lines, and PIP3 has been found to be overexpressed in PTC tumor specimens.

Genetic alterations involving RET have long been implicated in the molecular pathophysiology of sporadic PTC. The proto-oncogene RET encodes a receptor tyrosine kinase involved in cell survival. More than 15 translocations of the C terminus of RET with various N-terminus genes and their promoters have been found to form chimeric oncogenes in PTC. The most common of these are RET/PTC1 and RET/PTC3. The prevalence of these events has been estimated to be as high as 43% in PTC (12). Interestingly, RET/PTC rearrangements have been shown to signal through Ras, B-Raf, and Akt, once again highlighting the importance of these two signaling pathways in thyroid cancer.

1.3 TKIs and tumor angiogenesis

Tyrosine kinases are important cellular signaling proteins that have a variety of biological activities including cell proliferation and migration. Multiple kinases are involved in angiogenesis, including receptor tyrosine kinases such as the vascular endothelial growth factor receptor. Inhibition of angiogenic tyrosine kinases has been developed as a systemic treatment strategy for cancer. Angiogenesis, the growth of new vessels from preexisting vasculature, is a critical step in tumor progression (13). New blood vessels are required to support the growth of a tumor beyond the size of about 1–2 mm^3, to supply oxygen and nutrients to proliferating tumor cells and for metastasis formation. In the past two decades, inhibitors of angiogenesis have been developed for clinical use (14).

The lack of effective systemic treatments for advanced DTC combined with the knowledge that aberrant intracellular signaling plays a central role in thyroid cancer tumorigenesis present a unique opportunity for new targeted agents, many of which are multikinase inhibitors (MKIs) (Table 2).

	VEGFR	BRAF	PDGFR	KIT	RET	EGFR	Other
Sorafenib	+	+	+	+	+		FLT-3
Sunitinib	+		+	+	+		
Vandetanib	+				+	+	
Axitinib	+		+				
Motesanib	+		+	+	+		

Table 2: **Targets of TKIs for metastatic thyroid cancer.**

The MKIs exert their effects through competitive allosteric inhibition of the intracellular ATP-binding site of VEGFR-2 or other target kinases. VEGFR-1 and VEGFR-2 are expressed on tumor cells in thyroid cancer and other solid tumor malignancies. Common to all MKIs that have shown activity in DTC is VEGFR-2 inhibition.

1.4 Tyrosine kinase signaling in angiogenesis

In normal physiological circumstances, angiogenesis is well controlled by pro- and anti-angiogenic factors. Though, in cancer, this balance of pro- and anti-angiogenic factors is disturbed, resulting in the so-called 'angiogenic switch'. Tumor cells secrete a number of pro-angiogenic factors that stimulate the proliferation and migration of endothelial cells, resulting in the outgrowth of new capillaries into the tumor. The tyrosine kinase VEGFR is a crucial mediator in angiogenesis. The VEGFR family comprises three related receptor tyrosine kinases, known as VEGFR-1, -2, and -3, which mediate the angiogenic effect of VEGF ligands. The VEGF family encoded in the mammalian genome includes five members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF). VEGFs are important stimulators of proliferation and migration of endothelial cells. VEGF-A (commonly referred to as VEGF) is the major mediator of tumor angiogenesis and signals through VEGFR-2, the major VEGF signaling receptor (15). A second important growth factor involved in angiogenesis is the platelet-derived growth factor (PDGF). The PDGF family consists of at least four members: PDGF-A, PDGF-B, PDGF-C, and PDGF-D, which bind to two different receptors, known as PDGFR-a and -b (16). PDGFs facilitate recruitment of pericytes and smooth muscle cells and are important for maturation and stability of the vasculature (17). The 18 members of FGF family can be divided into six subfamilies and bind to seven main FGF receptors. FGF2 induces angiogenesis by stimulating migration and proliferation of endothelial cells. Furthermore, it supports proliferation of smooth muscle cells and fibroblasts (18).

Receptor tyrosine kinases are essential for the transduction of extracellular signals into the cell. A receptor tyrosine kinase monomer consists of an N-terminal extracellular ligand-binding domain, a transmembrane domain, and a C-terminal intracellular domain with tyrosine kinase activity. The kinase domain has a bilobar structure, with an ATP- binding cleft located between the N- and C-

terminal lobes. The ATP-binding site can be divided into three subregions: the adenine region, the sugar region, and the phosphate- binding region. The C-terminal lobe of kinases contains an activation loop and is marked by a specific amino acid combination at the start of the loop. This combination exists of the amino acids aspartic acid, phenylalanine, and glycine, abbreviated as D, F, and G, respectively, and is therefore called 'DFG motif'. The activation loop can adopt numerous conformations. In the 'out' conformation, the activation loop creates a hydrophobic pocket, nearby the ATP-binding cleft (Figure 1). This hydrophobic pocket is important for a subgroup of tyrosine kinase inhibitors.

Ligand binding to the extracellular domain of the receptor promotes receptor dimerization, resulting in autophosphorylation of specific tyrosine residues of the cytoplasmic kinase domain. Besides these phosphorylation sites for regulation of their own kinase activity, other phosphorylation sites of kinases are being used to control protein interactions. The activated receptor recruits interacting proteins that bind to certain phosphorylation sites. Recruited and phosphorylated signaling proteins are subsequently able to phosphorylate other proteins. Activation of signaling pathways eventually leads to biological responses. Biological responses include cell activation, proliferation, differentiation, migration, survival, and vascular permeability.

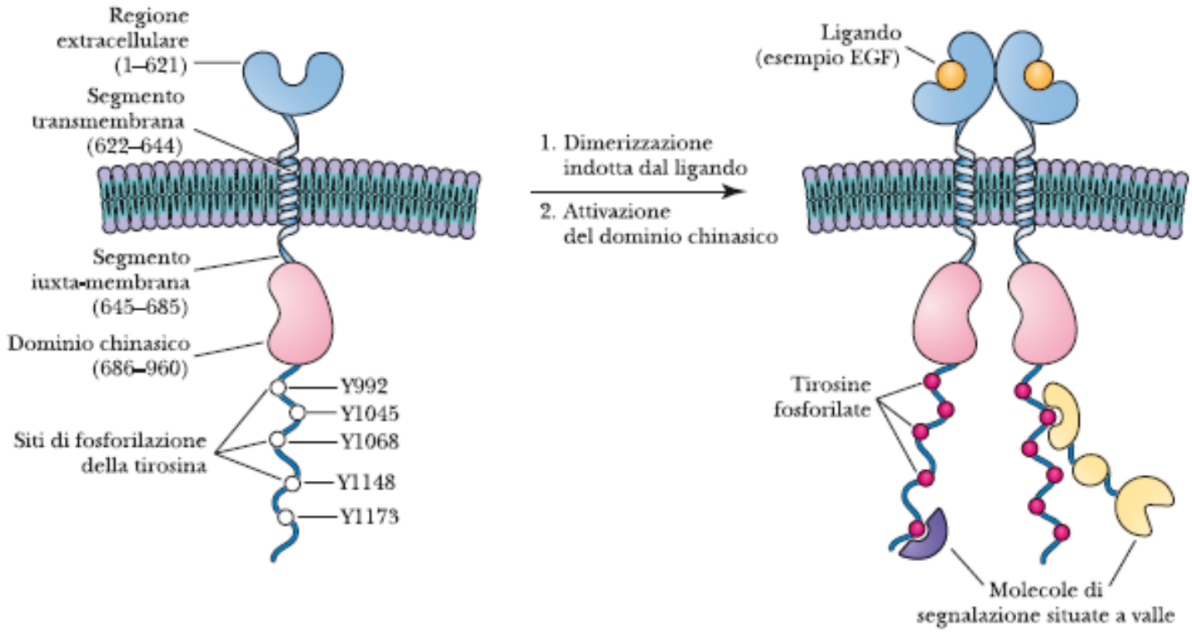

Figure 1: **Structure and dimerization of a receptor tyrosine kinase (example of EGF receptor).**

Stimulation of VEGFRs and other tyrosine kinase receptors causes massive activation of signaling pathways in endothelial cells. Signaling molecules downstream of receptor tyrosine kinases not only include tyrosine kinases, but involves other signaling proteins as well including serine/ threonine kinases and G-proteins. Important signaling molecules recruited to tyrosine kinase receptors comprise proteins with a Src homology 2 (SH2) domain. Association of a phosphorylated tyrosine kinase receptor with a SH2 domain-containing protein results in the phosphorylation and activation of this effector protein. In addition, the binding of this SH2 domain-containing protein to the receptor serves as a docking site for other signaling molecules. Phospholipase C-c (PLCc) is an SH2 domain-containing protein that is frequently involved in signaling by VEGFRs. PLCc phosphorylates protein kinase C (PKC), which subsequently phosphorylates a range of kinases. Phosphorylation of MEK (mitogen-activated protein kinase (MAPK) and extracellular-signal-regulated kinase (ERK) kinase) by PKC stimulates the p42/44 MAPK pathway. Phosphorylated MAPK, a serine/threonine kinase, activates various transcription factors and is known to

regulate cell proliferation (Figure 2).

Another signaling molecule involved in the MAPK cascade is the growth factor receptor-bound protein 2 (Grb2). Grb2 contains SH2 and SH3 domains and is able to activate the G-protein Ras via association with the ATP/ADP exchange factor mammalian Son-of-sevenless (Sos). The Ras protein can bind to and phosphorylate Raf, which in turn can activate the MEK/MAPK pathway. The Ras/Raf pathway is a classical pathway in activation of MAPK and is involved in signaling of many tyrosine kinase receptors, for example, the epidermal growth factor receptor (EGFR). However, activation of the Ras/Raf pathway plays a minor role in VEGFR signaling.

An adapter molecule that is important in VEGFR- mediated signaling is the SH2 and b-cells (Shb) protein. Interaction of Shb with a specific phosphorylation site of VEGFR-2 activates phosphatidylinositol 3-kinase (PI3K). PI3K and its downstream activated serine/threonine kinase Akt/protein kinase B (PKB) are involved in several important processes of angiogenesis, including endothelial cell migration, proliferation, and survival, as shown in Fig. 2B. Activation of Akt/PKB requires generation of phosphatidylinositol 3,4,5-triphosphate (PIP3) by PI3K- mediated phosphorylation of phosphatidylinositol 4,5- biphosphate (PIP2). Akt/PKB stimulates proliferation and survival by the activation or inhibition of a variety of substrates. Akt/PKB phosphorylates and inhibits the pro-apoptotic protein BAD (Bcl-2 associated death promoter) as well as GSK3 (glycogen synthase kinase 3). Akt/ PKB is also able to activate the mammalian target of rapamycin (mTOR) and its downstream p70S6K which are regulators of cell proliferation and survival. In addition, Akt/PKB enhances cellular proliferation through activation of nuclear factor-jB (NF-jB). Furthermore, Akt/PKB is able to stimulate vasodilation, vascular remodeling, and angiogenesis, through phosphorylation of endothelial nitric oxide (NO) syntheses (eNOS). Last, the PI3K pathway seems to be involved in endothelial cell migration.

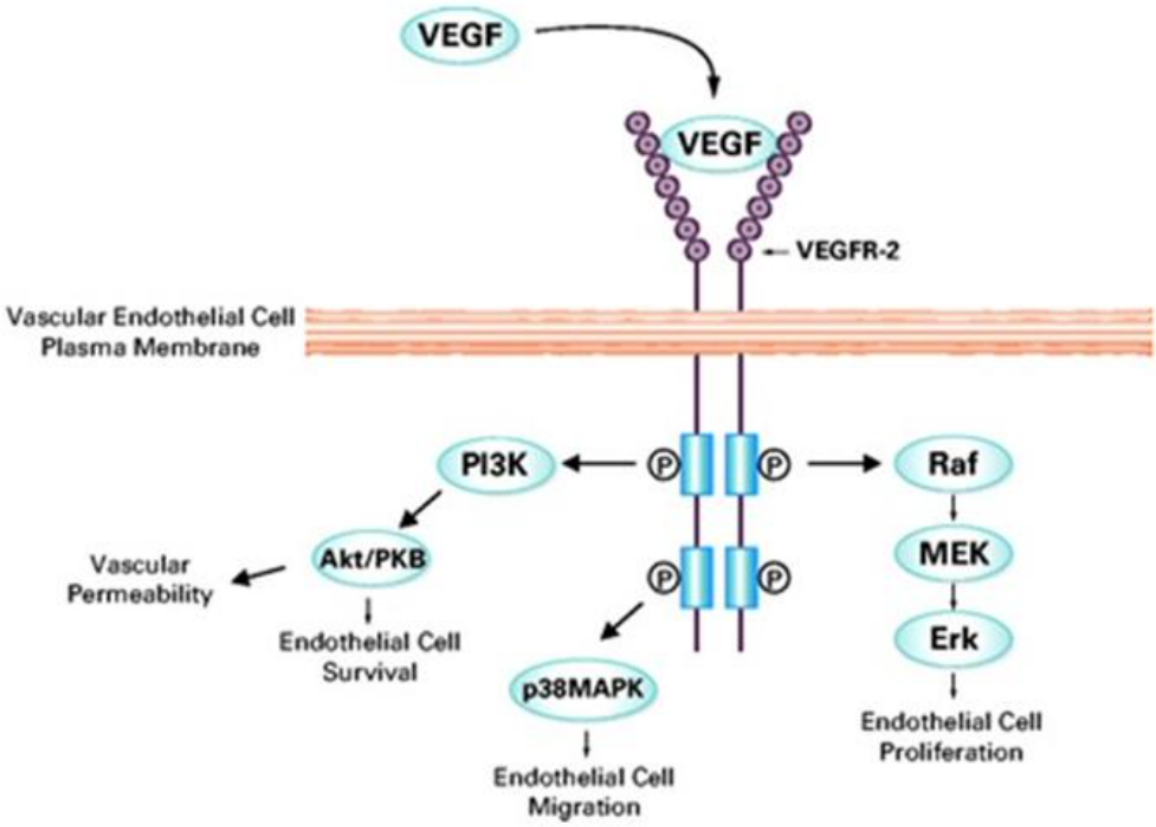

Figure 2: **Signal transduction pathways and biological processes mediated by VEGF receptor focused on angiogenesis.**

1.5 Toxicities of TKIs

Selective inhibitors may induce toxicities, because their target kinases are not differentially expressed by endothelial cells. Angiogenesis inhibitors are intended to target activated tumor endothelium. Initially, these agents were not expected to target normal vasculature, because most blood vessels remain quiescent during adulthood. However, under normal physiological circumstances, growth factor signaling in endothelial cells seems important for their survival and maintenance of vascular integrity. Inhibitors of angiogenesis are capable of affecting signaling pathways in endothelial cells and might elicit toxicities as a result of decreased endothelial cell renewal capacity.

Apart from kinase inhibitors with 'off-targets' activities, downstream signaling pathways of target kinases may also be involved in the development of toxicities. One kinase is able to activate several downstream signaling pathways. By inhibition of a kinase with a tyrosine kinase inhibitor, a whole spectrum of signaling pathways can be deactivated. This might result in a reduction in specific biological outcomes which are not intended to adjust. It has been shown that specific kinases are involved in the normal physiology of certain organs such as the thyroid gland. It has been suggested that specific toxicities, like fatigue, might be related to interference of these inhibitors with the normal function of these organs (19).

1.6 TKIs and thyroid dysfunction

Thyroid dysfunction associated with TKIs is common. The prevalence of thyroid dysfunction (hypo- or hyperthyroidism) related to a varies considerably from about 3.1% to 100% depending on the type of molecule, the dose administered, the types of thyroid monitoring and the recording accuracy of these events (19, 20). In a meta-analysis, the relative risk of hypothyroidism was 3.59 [95% CI: 2.40-5.38; $p<0.0001$] (20). The probability of developing TKI-induced dysthyroidism depends on the patient's background (risk is higher in female and older patient groups), the existence of associated thyroid disorder, the duration of TKI exposure and the molecule. On the other hand, the type of cancer treated does not seem to be a factor modifying the risk of thyroid side effects. Iatrogenic thyroid disorder related to TKIs is mainly caused by destructive thyroiditis, probably due to vascular damage.

Sunitinib-induced thyroid toxicity has been the most studied clinical model for 10 years. Desai et al, have conducted a prospective study with 42 patients treated by sunitinib. Persistent primary hypothyroidism occurred in 15 (36%) patients. Six of 15 (40%) hypothyroid patients had suppressed TSH concentrations before developing hypothyroidism suggesting induced thyroiditis through a destructive

mechanism (21). Such biphasic thyroiditis pattern was reported from 13,3 to 87,5% of cases. First, thyrotoxicosis is reported, accompanied by a transient increase in thyroglobulin associated with TKI-induced cell lysis, and then hypothyroidism develops secondarily. This sequence is repeated at each cycle when an intermittent on/off pattern is prescribed. When several cycles of treatment are performed, a decreased vascularization of the thyroid parenchyma is reported with thyroid hypotrophy which will eventually be responsible for permanent hypothyroidism if the treatment is prolonged. (21-24). The thyroiditis reported with anti-angiogenic TKIs is therefore the consequence of devascularization of the thyroid vesicles, which represent the functional units of the thyroid. The vascularization abnormalities and the resulting cellular hypoxia are related to capillary regression mediated by the anti-VEGFR effect. There is both functional and structural damage. Indeed, VEGF-A/VEGFR2 signaling is directly involved in all fenestrated capillaries (which allows the introduction of iodine and the secretion of thyroid hormones through the endothelial cells) and in the trophic development of the thyroid tissue, according to the work conducted in murine models (25). Therefore blocking this signaling pathway also leads to a decreased synthesis of thyroid hormones. Other intrathyroid mechanisms have been suspected in the occurrence of hypothyroidism under TKI, such as the inhibition of iodine uptake by thyrocytes and the inactivation of thyroperoxidase (26). These hypotheses were subsequently invalidated because this functional impairment was a logical consequence of the ischemia and tissue damage induced by TKIs. Regarding the central nervous system, it is also assumed that some TKIs, by decreasing the production of nitric oxide, could decrease the secretion of TRH by the paraventricular nucleus of the hypothalamus and thus decrease the secretion of TSH by the thyrotrophic pituitary cells (27).

Additional pathophysiological hypotheses have been put forward to explain the worsening hypothyroidism of the thyroidectomized subject, involving alteration of the transport and metabolism of thyroid hormones with certain TKIs.

TKIs also have effects on the peripheral metabolism of thyroid hormones regardless of their own thyroid toxicity. In thyroidectomized patients treated with TKIs, there is an increased demand for thyroid hormone. Indeed, TKIs such as sunitinib or sorafenib increase the activity of type 3 deiodinase (as evidenced by the decrease in T3/T4 and T3/rT3 ratios) resulting in hypothyroidism because of lower tissue availability of the active hormone T3, locally inactivated in T2 or rT3. Finally, other TKIs such as imatinib, bosutinib and dasatinib, but also sunitinib, can inhibit the transporter of MCT8 thyroid hormones (monocarboxylate transporter) across the plasma membrane, reducing the supply of T3 to peripheral tissues but also centrally, in the thyrotropic cell (28). Thyroid hormones exert their action by binding to specific nuclear receptors that are heterodimerized with a retinoic acid receptor. Sunitinib, by binding to retinoic acid receptors, could prevent this heterodimerization and thus inhibit the expression of target genes (29) (Figure 3).

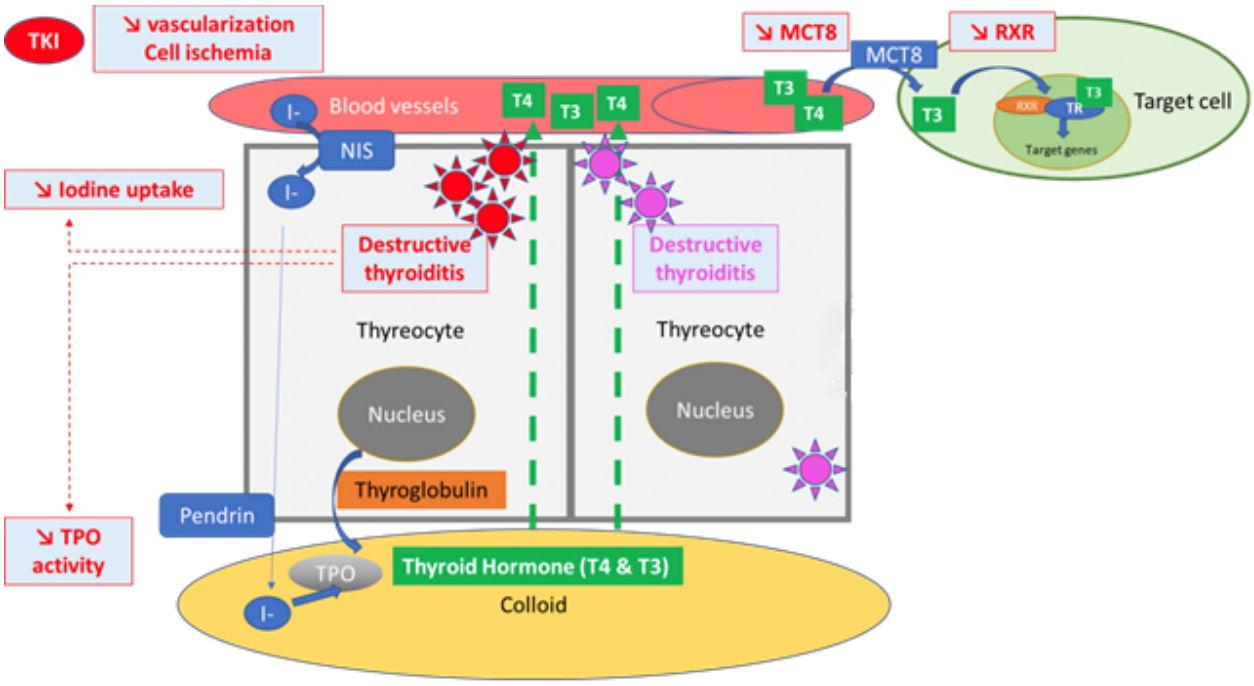

Figure 3: **The pathophysiological mechanisms of dysthyroidism under TKIs therapy** (adapted from Jannin A et al Critical Reviews in Oncology / Hematology 141 (2019) 23–35).

The symptoms and physical signs of dysthyroidism under TKI therapy are often discrete. Paraclinical evaluation is essential to establish the diagnosis of iatrogenic thyroiditis and eliminate a differential diagnosis by listing the patient's comorbidities and confounding factors (nutritional status, treatments, injections of iodinated contrast agents). During the thyrotoxicosis phase, TSH is low and free T4 increased (or normal in cases of subclinical hyperthyroidism). During the hypothyroid phase, TSH is high and free T4 is low (or normal in cases of subclinical hypothyroidism). During this phase, TPO-Abs and/or Tg-Abs are present, but generally low (25% of patients under TKI therapy).

The thyroid gland has a different ultrasound appearance in thyroiditis under TKI. A hypoechoic, heterogeneous appearance and decreased vascularization are initially observed by Doppler, then a progression to thyroid atrophy.

In the phase of biological hyperthyroidism, in case of diagnostic doubt with Graves' disease or functional nodular dystrophy, a thyroid scintigraphy may be performed.

No predictor of the occurrence of dysthyroidism has been clearly identified to date for TKIs. It is recommended to measure the TSH/free T4 levels before starting treatment, and every 3 to 4 weeks during the first 6 months as changes in free T4 levels precede the changes in TSH by 3 to 6 weeks. After this period, the laboratory tests may be conducted every 2-3 months, and simplified by the TSH measurement alone.

Screening for anti-thyroid autoimmunity (TPO-Abs, Tg-Abs, TRAbs) before initiation of TKIs is not recommended. A history of thyroid disorder does not contraindicate initiation of TKI therapy. In hypothyroid patients in these classes of anticancer drugs, adjustment of the replacement therapy may be necessary; it will be performed in connection with the patient's endocrinologist.

The occurrence of thyroid dysfunction, usually grade 1 or 2, does not contraindicate the continuation of TKI therapy.

During the thyrotoxicosis phase, a symptomatic treatment will be initiated including low-dose non-cardioselective β-blockers such as propranolol, in the absence of contraindication. Treatment with synthetic antithyroid drugs will be initiated only in cases of Graves' disease, toxic nodular goiter or solitary toxic nodules detected during this monitoring.

Given the risk of progression to hypothyroidism in the weeks following thyrotoxicosis, regular monitoring of TSH and free T4 is required. Hyperthyroidism is almost always resolving, followed or not by a hypothyroidism phase. In the hypothyroid phase, the β-blocker treatment is of course discontinued and a supportive treatment with levothyroxine may be started. A follow-up TSH test will be performed 6 weeks after the prescription of Levothyroxine. Patients

with already substituted hypothyroidism should have the TSH levels carefully monitored in order to adjust the levothyroxine doses if necessary (Figure 4).

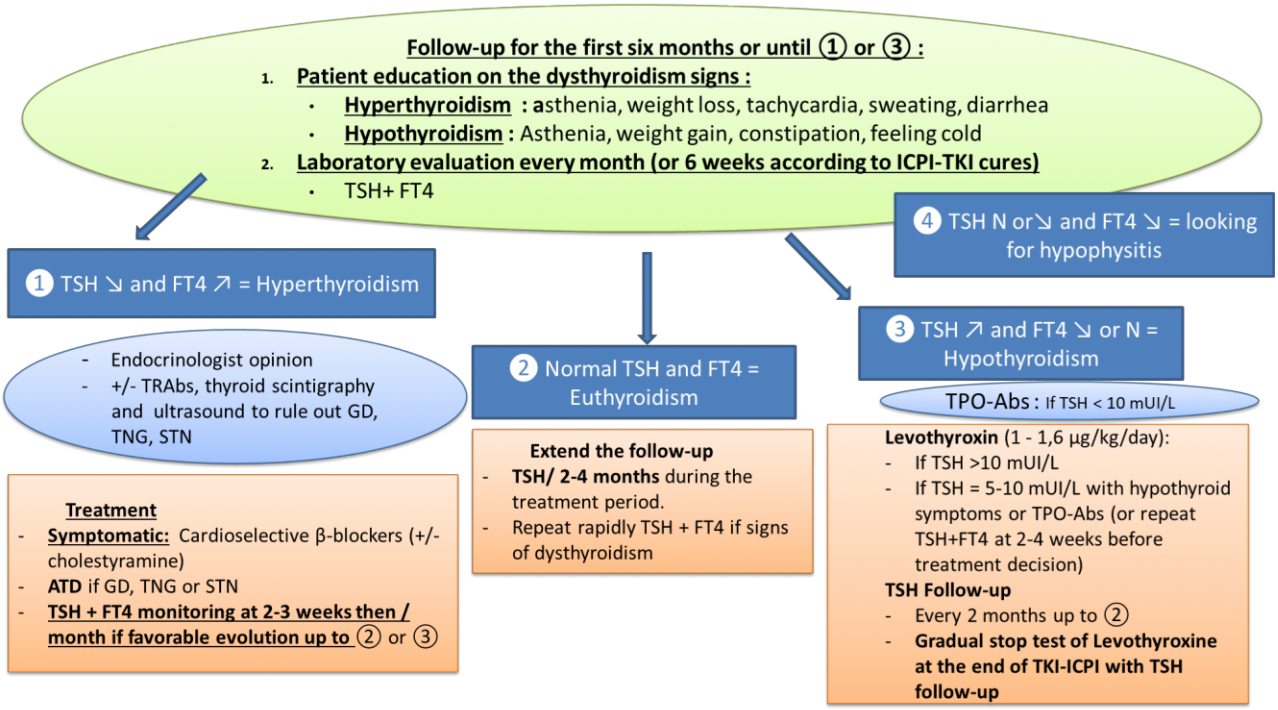

Figure 4: **Treatment of dysthyroidism during TKIs treatment** (adapted from Jannin A et al Critical Reviews in Oncology / Hematology 141 (2019) 23–35).

1.7 Thyroid hormone and heart

Thyroid hormones have central regulatory actions in the cardiovascular system, particularly in the heart. Changes in plasmatic or peripheral thyroid hormone levels are associated with significant alterations in cardiovascular function.

Thyroid hormones have both direct and indirect effects on cardiac function in rat. These effects are mediated through genomic and nongenomic mechanisms. T3 binds to a specific nuclear receptor which regulates the transcription of various genes with important roles in cardiovascular function (30). The nongenomic effects are mainly related to cell membrane transport of calcium and other ions. In addition to the heart, T3 also has effects on the peripheral circulation. These impact cardiovascular hemodynamics, cardiac filling, and myocardial

contractility (31, 32). Thyroid hormones upregulate the expression of genes encoding sodium/potassium-transporting ATPases, increase the transcription of myosin heavy chain (MHC) α gene, and decrease that of MHC β gene, resulting in an increased velocity of contraction (33-34). They also increase the transcription of calcium ATPase protein of the sarcoplasmic reticulum (SERCA2a) and downregulate the transcription of phospholamban (PLN), increasing the velocity of diastolic relaxation (34). T3 decreases cardiac fibrosis through repression of collagen gene expression and induction of metalloproteinase expression. Thyroid hormones also possess positive inotropic properties through increased expression of β1-adrenergic receptors and modulation of ionic channels (33) (Table 3).

Gene	Transcription	Effect
Myosin heavy chain α (MHC α) Myosin heavy chain β (MHC β)	↑ ↓	↑ velocity of contraction
SERCA 2 Phospholamban (PLN)	↑ ↓	↑ velocity of diastolic relaxation
Collagen Metalloproteinase	↓ ↑	↓ cardiac fibrosis
β1 adrenergic receptor	↑	↑ velocity of contraction ↑ heart rate

Table 3: **Genomic effects of thyroid hormones.**

Regarding indirect effects, thyroid hormones activate sodium, potassium, and calcium membrane channels, have effects on the mitochondrial membrane and mitochondriogenesis, and are involved in several signaling pathways of cardiomyocytes and vascular smooth muscle cells. Thyroid hormones induce production of endothelial nitric oxide and subsequent vasodilatation through the activation of phosphatidylinositol 3kinase (PI3K)/serine/threonine protein kinase (AKT) signaling pathways. By increasing calcium reuptake within the arterioles, thyroid hormones also promote smooth muscle relaxation. Both these effects and

the direct repression of PLN expression lead to a reduction in systemic vascular resistance (34). In sum, thyroid hormones improve systolic and diastolic function, decrease systemic vascular resistance through vasodilation and increase cardiac output through positive inotropic and chronotropic effects. (31).

Hypothyroidism has many cardiac effects opposite to those seen in hyperthyroidism. Hypothyroidism and subclinical hypothyroidism produce similar qualitative cardiovascular alterations that only differ in their extent. Kisso et al. (35) induced overt hypothyroidism in hypertensive rats by treating them with propylthiouracil (PTU). They found that left ventricular (LV) diameters in systole and diastole were increased, while wall thickness, ejection fraction, heart rate, and systolic blood pressure were decreased in these rats. Furthermore, thyroid hormone dysfunction (hypothyroidism) in previously hypertensive rats led to systolic dysfunction and LV dilation (36). In hypothyroidism, heart rate and contractility are reduced, and peripheral vascular resistance is increased, leading to a reduction in cardiac output (Table 4) (37).

Hyperthyroidism and sublinical hyperthyroidism	**Hypothyroidism and sublinical hypothyroidism**
↑ cardiac output	↓ cardiac output
↑ heart rate	↓ heart rate
↑ contractility	↓ contractility
↓ systemic vascular resistance (↑ NO)	↑ systemic vascular resistance (↓ NO)
↑ carotid intima-media thickness	↑ carotid intima-media thickness
↑ systolic blood pressure	↑ diastolic blood pressure
Cardiac hypertrophy	Cardiac fibrosis
Heart failure	Heart failure
Increased arrhythmic risk	Hypercholesterolemia

Table 4: **Cardiovascular effects of hyperthyroidism and hypothyroidism**

Overt hypothyroidism is accompanied by carotid intima–media thickening. It is also associated with other atherosclerotic risk factors such as hypercholesterolemia, diastolic hypertension, and reduced production of nitric oxide. These effects are reversible with thyroid hormone supplementation.

Subclinical hypothyroidism (SCH) is most frequently associated with diastolic dysfunction due to impaired ventricular filling and relaxation. Nevertheless, SCH is also associated with systolic dysfunction, which is reversed with thyroid hormone replacement therapy. Similarly to overt hypothyroidism, SCH increases systemic vascular resistance and arterial stiffness by impairing relaxation of vascular smooth muscle cells and by reducing nitric oxide availability. Patients with SCH present with increased systolic and diastolic blood pressures and total cholesterol concentrations.

Population studies have found conflicting evidence concerning the association between SCH and cardiovascular morbidity and mortality. Some studies concluded that SCH does not increase cardiovascular mortality, while others suggested it does. There are no randomized clinical trials to show whether or not thyroid hormone replacement therapy improves cardiovascular morbidity and mortality in SCH patients. Several levothyroxine supplementation therapy studies in SCH individuals showed improvements in cardiovascular function (improved LV function, vascular endothelial function, and decreased atherogenic lipid particles, among others). One study found that patients (aged < 70 years) with high TSH levels treated with levothyroxine had fewer cardiovascular events when compared with untreated patients (37). Due to the aforementioned lack of evidence and according to international guidelines, treatment should solely be considered in patients with more severe disease (TSH levels > 10 mU/l), or aged < 70 years, or that present with symptoms of hypothyroidism (38).

Thyroid hormones can vary at a local level independently of serum thyroid hormone levels. D3 can decrease thyroid hormone signaling specifically in the

cardiac tissue and may contribute to the regulation of cardiac thyroid function during heart failure progression (39). Heart failure is also associated with downregulated thyroid hormone nuclear receptors, which may worsen cardiac function even further. Wassen et al. demonstrated that rats with right ventricular hypertrophy have increased D3 activity in the RV but equal D3 activity in the LV when compared with controls. Moreover, rats with right ventricular hypertrophy that progressed to heart failure had even higher D3 activity when compared to rats with compensatory hypertrophy. The authors only found changes in serum thyroid function (decreased levels of T3) in rats that progressed to heart failure. The increase in D3 activity may represent a maladaptive process, as stable cardiac hypertrophy has less D3 activity than decompensated right ventricular hypertrophy (39). Besides upregulation of D3, there are other altered processes that can contribute to locally impaired thyroid function in cardiac tissue: decrease in thyroid hormone uptake, changes in the expression of thyroid hormone receptors in cardiomyocytes, altered peripheral T4 to T3 conversion, reduced T3 production by inhibition of D2, and changes in thyroid hormone membrane transporters.

Data from epidemiological studies supports a higher risk of heart failure and a worse prognosis in heart failure patients with low levels of TH (37). In addition, animal studies and small clinical studies suggest that TH supplementation may improve cardiac function in heart failure. (40).

1.8 Thyroid hormone action and deiodinases

Thyroid hormones (THs), triiodothyronine (T3), and thyroxine (T4), are involved in different biological processes as cell growth, development, differentiation, and the regulation of metabolism and homeostasis (41).

In the bloodstream, the steady-state level of TH concentration is regulated by the hypothalamic-pituitary-thyroid (HPT) axis. Hypothalamic thyroid releasing-hormone (TRH) stimulates thyrotrophic cells in the anterior pituitary to produce thyroid

stimulating-hormone (TSH). In turn, TSH induces the production of pro-hormone thyroxine (T4) and-to a lesser extent-the active form triiodothyronine (T3) by the thyroid gland (42). Secreted THs are released into the circulation and carried bound to proteins such as thyroxin binding globulin, transthyretin or serum albumin (43).

Besides systemic regulation, the TH concentration in target tissues can differ remarkably depending on local TH metabolism, which ultimately regulates target gene expression.

To exert its functions, TH must overcome several check-points, namely TH transporters, TH-metabolizing enzymes (deiodinases), TH receptors (TRs), and their interactions with co-repressors and co-activator (44). Once TH enters the bloodstream, a low amount of TH, not bound to circulating transport proteins, is free to act on target cells.

The access of TH to the intracellular compartment is mediated by four different families of TH-transporting proteins that have been shown to be involved in the traffic of iodothyronines across the cell membrane (45-47) These transporters are differentially expressed in tissues in a developmental and cell-type-specific fashion and, while most of them accept a variety of ligands, others have elevated substrate specificity (45, 48).

Among the most important thyroid hormone transporters are MCT8 and MCT10. The monocarboxylate 8 (MCT8) is probably the most relevant transporter as mutations in the MCT8 protein have been associated with variable levels of mental retardation in humans combined with lack of speech development, muscle hypotonia and endocrine dysfunctions (45, 49).

MCT8 transports both T4 and T3 and is expressed in liver, muscle, kidney and in many brain areas (50, 51), whereas the monocarboxylate 10 (MCT10) preferentially transports T3 instead of T4 and is expressed in kidney, liver and muscle (45). Once transported into the cell, thyroid hormones can be metabolised by outer or inner ring deiodination through the iodothyronine deiodinases, a selenocysteine-containing

enzyme family consisting of three types: type 1 (DIO1), type 2 (DIO2) and type 3 (DIO3) (Figure 5) (52).

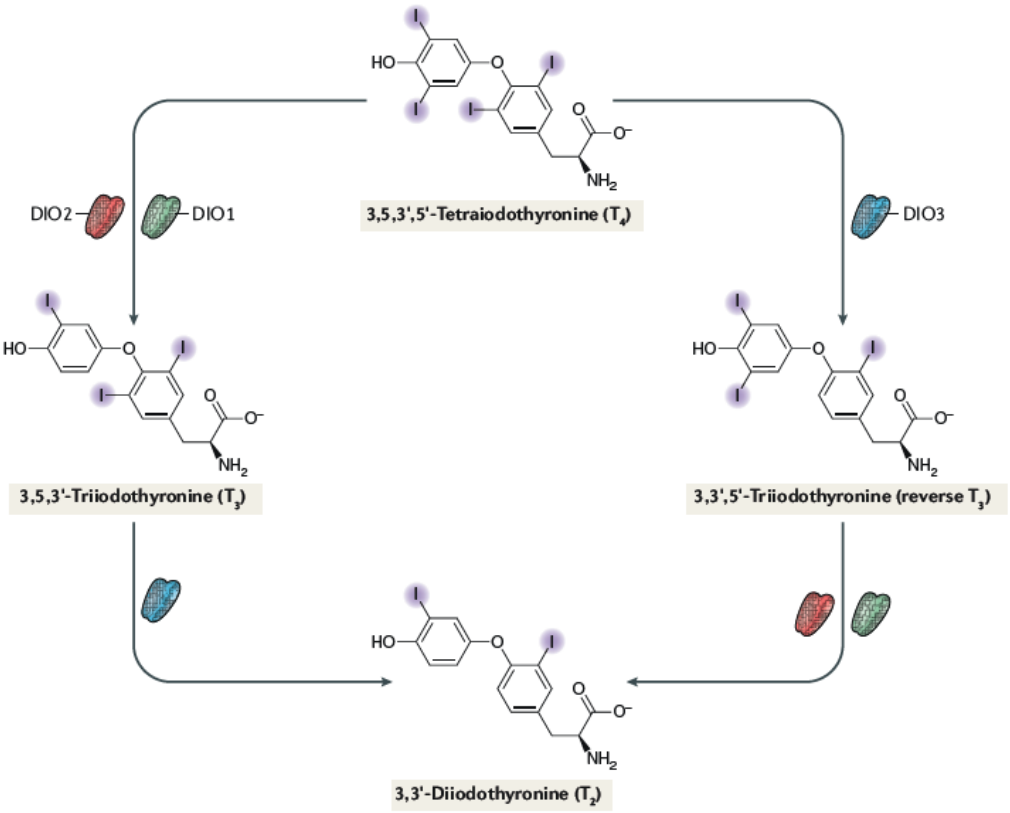

Figure 5: **Deiodinase thyroid hormone action**.
The pro-hormone T4 is activated by monodeiodination of the phenolic thyronine ring consequent to the action of DIO1 or DIO2 to form T3. Deiodination of the tyrosyl ring by DIO1 or DIO3 inactivates T4 and T3. Additional monodeiodination reactions generate reverse T3 (through DIO3-mediated T4 deiodination) and T2 (through DIO1-mediated and DIO2-mediated T3 deiodination). Reverse T3 cannot bind to the receptor and is thus considered an inactive metabolite. T2 is not involved in the genomic action of T3, but it might exert various metabolic effects.

All deiodinases are membrane-anchored proteins of 29–33 kDa that share substantial sequence homology, catalytic properties and contain the selenocysteine (Sec) aminoacid as the key residue within their catalytic center (53). All the three deiodinases are homodimers whose dimerization is required for full catalytic activity (54, 55). The unique feature of selenoproteins in general, and deiodinases in particular, lies in the recoding of the UGA codon from a stop codon to Sec-insertion codon by the presence of the SECIS element in the 3′ UTR of the respective mRNAs. Iodinated contrast agents such as iopanoic acid inhibit all three deiodinases, while propylthiouracil is a relatively specific inhibitor for DIO1 (53).

DIO1 is localized in the plasma membrane and is able to exert both activating and inactivating functions. DIO2 is localized in the endoplasmic reticulum and the DIO2 enzyme is considerably more efficient than DIO1: it catalyses the removal of an outer ring iodine atom from the pro-hormone T4 to generate the physiologically active product T3 (56, 57). DIO1 is expressed in the thyroid, liver and kidneys, whereas DIO2 contributes to T3 production in the central nervous system, thyroid, skeletal muscle and brown adipose tissue (58).

DIO3 is localized in the plasma membrane and is the terminating enzyme, which irreversibly inactivates T3 and prevents T4 activation by catalysing the removal of an inner ring iodine atom, generating inactive metabolites that do not interact with T3 receptors, reverse triiodothyronine (rT3) and 3,3'-diiodothyronine (T2), respectively (59, 60) (Figure 6).

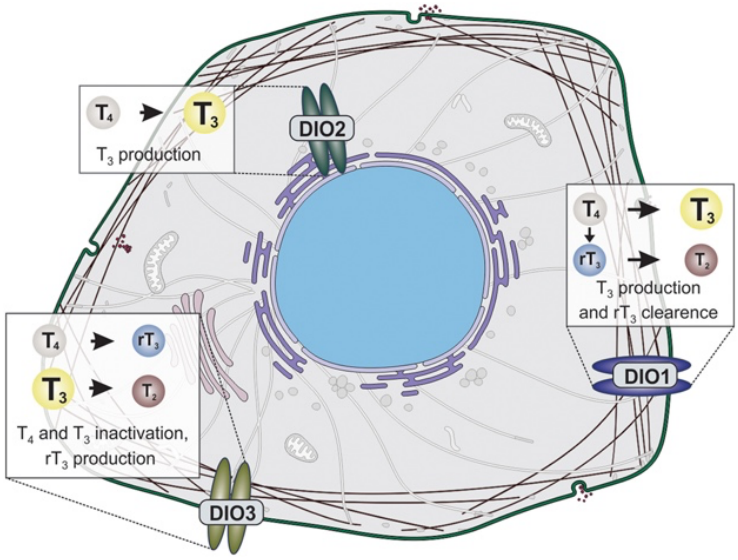

Figure 6 **Types 1, 2, and 3 iodothyronine selenodeiodinases.**
DIO1 is localized in the plasma membrane and is able to exert both activating and inactivating functions of TH.
DIO2 is localized ~~is localized~~ in the endoplasmic reticulum and contributes to T3 production from the pro-hormone T4. DIO3, the TH-inactivating enzyme, is localized in the plasma membrane.

DIO3 is highly expressed in placenta and plays an important role during embryonic development protecting the fetus from excessive exposure to active thyroid hormone. DIO3 is also expressed in the brain and skin, but in adult healthy tissues expression levels are very low.

However, under specific pathophysiological conditions, its expression can be induced in other tissues (61) and is correlated with hyperproliferation conditions as cancer.

Therefore, depending on whether the deiodination occurs on the inner (IDR) or outer ring (ODR) of the iodothyronine substrate, the deiodination results in activating pathway or inactivating pathway.

Currently, tissue thyroid hormone concentrations are thought to be determined by the balance between deiodinases present in the tissue rather than by serum TH concentrations alone. Thus, the TH-inactivating enzyme impedes thyroid hormone access to specific tissues at critical times and prevents TH receptor saturation (62).

Once the active hormone T3 is present inside the cells, the classical mechanism of action of THs is mediated by the binding of T3 to nuclear receptors (TRs), a family of ligand-dependent transcription factors that enhance or inhibit the expression of target genes by binding to specific DNA sequences, known as TH response elements (TREs).

Thyroid hormone receptors exist in two isoforms, TRα and TRβ, which are encoded by the THRA and THRB genes, respectively (44).

The binding of T3 to TRs promotes a conformational change that induces the exchange of corepressors for coactivators, thus leading to gene transcription on responsive genes, a process known as the genomic effect of thyroid hormones (63, 64)

Besides the genomic action of THs, a second mechanism of TH actions is constituted by the non-genomic effects of thyroid hormones, which involves the binding of T3 or T4 to cellular proteins and interactions of thyroid hormone receptor with cellular partners (44). Importantly, although these cytosolic interactions of thyroid hormone or thyroid hormone receptor are collectively called non-genomic actions, they might culminate in transcriptional activation of target genes (65).

2 Aim of the project

Thyroid Hormone (TH) is a pleiotropic agent that has a central importance in tissue homeostasis such as in cardiac function. At tissue level, TH action is regulated by the expression and activity of three selenodeiodinases, which catalyze TH activation (D1 and D2) and catabolism deiodinases (D3). The deiodinase system is often altered in some pathological conditions or influenced by drugs. Several studies have demonstrated that TKIs, expecially vandetanib, cause hypothyroidism (23). However, the molecular mechanisms that cause hypothyroidism vandetanib-induced as well as the ECG alterations are not clear.

My PhD project aimed to understand how vandetanib affects local tissue-specific TH concentration in the heart and whether D2 action is a target of vandetanib and could cause thyroid metabolism disfunctions.

Our hypothesis is that dissecting the influence of TKIs in the deiodinase activity is crucial for understand the mechanisms by which vandetanib causes tissue and systemic thyroid dysfuncion in patients under TKIs treatment .

3 Results

3.1 D2 expression in mouse heart

Several studies indicated that type 2 deiodinase is expressed in human and mouse heart (66). Tabula Muris (67), a compendium of single cell transcriptomic data from the model organism Mus musculus comprising more than 100,000 cells from 20 organs and tissues, indicates that D2 expressing cells in the myocardium are located in the atrioventrucular node. Particularly, D2 RNA is highly enriched in fibroblasts (Figure 7).

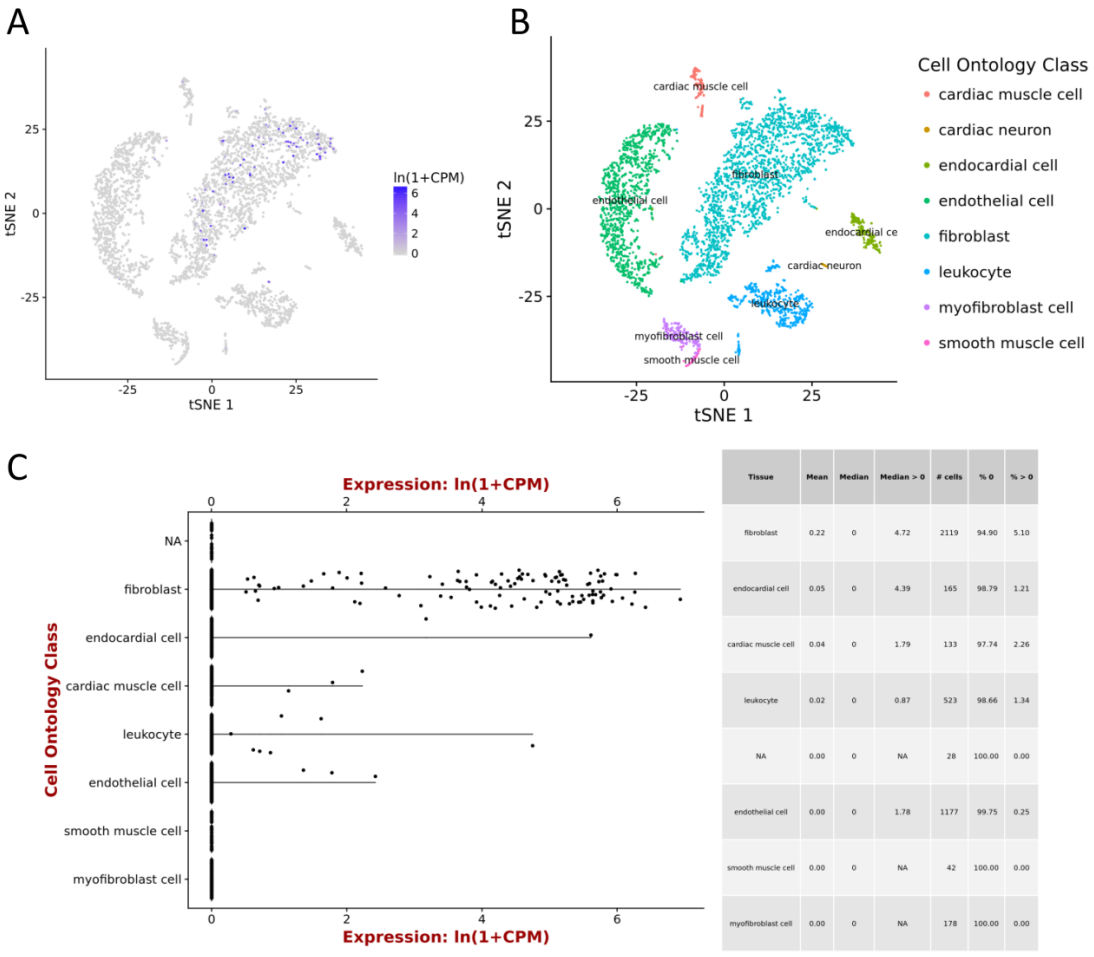

Figure 7: **D2 expression in mouse heart from Tabula Muris**. A) D2 positive cells, B) Cell ontology classification, C) D2 transcript distribution in different cell type.

Since no antibodies are available to detect D2 protein expression by western blot or by immunohistochemical analyses, we took advantage of a mouse model for D2 protein

detection. To identify the specific region in the heart where D2 is expressed we used a new mouse model that allow us to specifically track and visualize D2 expressing cells: D2CRE^{ert}; Rosa26zsGreen. These mice present a CRE^{ert} recombinase enzyme localized downstreem of *Dio2* promoter gene and in Rosa26 locus possess a CAG promoter *followed by a loxP*-flanked STOP transcription box designed to prevent the transcription of the downstream enhanced green fluorescent protein variant ZsGreen1 (see below). In presence of Tamoxifene, CRE expressing cells remove the STOP transcription box activating high expression of ZsGreen1. These mice are useful as a Cre reporter strain; expressing the enhanced green fluorescent protein, ZsGreen1, following Cre-mediated recombination. Thanks to these mice it was possible to determine that most of the D2 expressing cells were located in specific and restricted cardiac regions (Figure 8) such as VAT, atrioventricular node and cardiac valve.

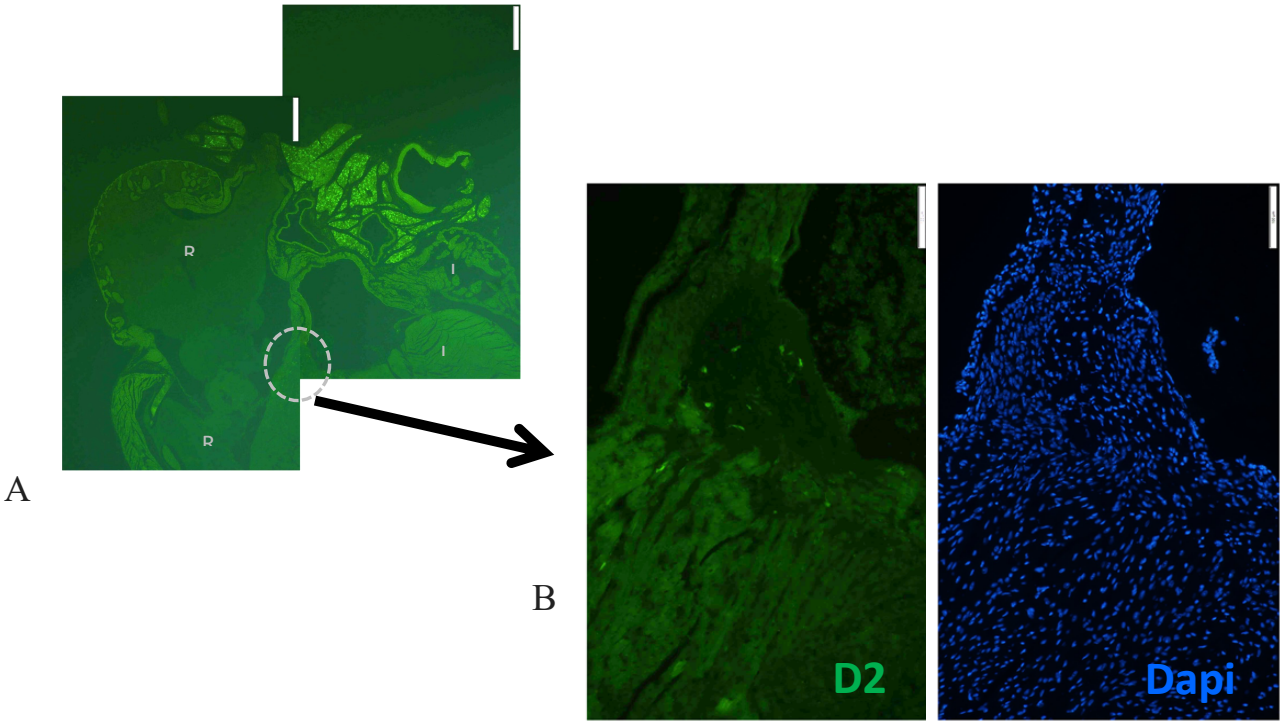

Figure 8: **D2 expression in atrioventicular node.**

Image of autofluorescence of D2 cell (Green cells) in heart section of Rosa26 D2 – Green mice. The 4X (A) and 20X (B) acquisitions show that D2 (Green cells) is expressed in a group of cells localized in the atrioventricular node.

3.2 Effects of Vandetanib on D2 expression

3.2.1 Effects of Vandetanib on D2 expression in mouse heart

To investigate whether Vandetanib affects D2 expression, we treated 9 male mice with Vandetanib (40 mg / prokg / day) and 6 male mice with placebo (physiological solution). Vandetanib was administred for 20 days in the drinking water.

To determine D2 expression we extracted mRNA from collected hearts after treatment and measured the levels of D2 mRNA by Real Time PCR.

Interstingly, we observed that after 20 days of treatment with Vandetanib, D2 mRNA is downregulated in the heart of treated mice compared with control mice.

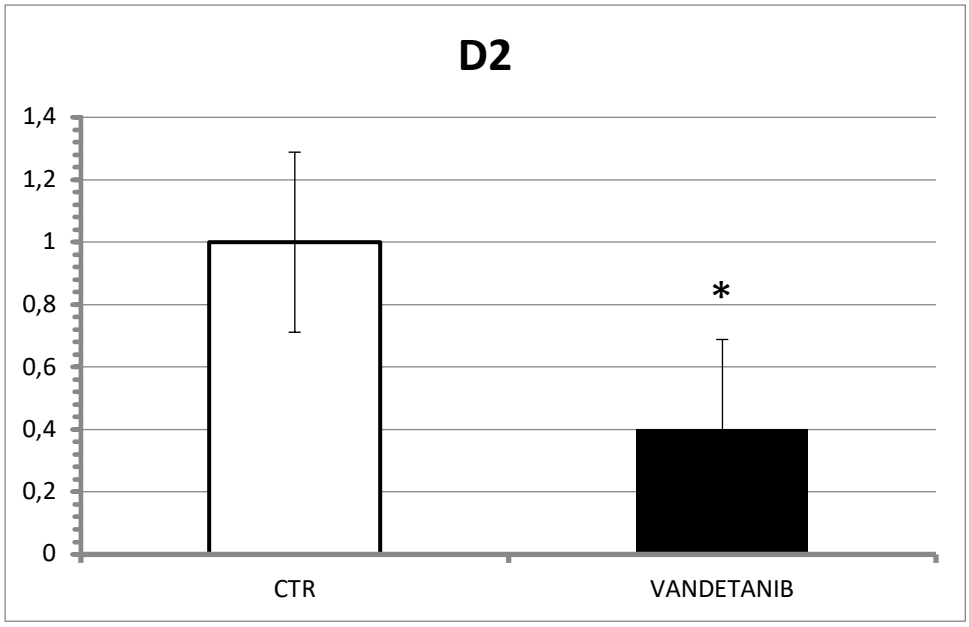

Figure 9: **D2 expression in Vandetanib treated mice.**
D2 mRNA is expressed at lower level in treated heart mice. Data is significant * p<0,05

3.2.2 Vandetanib effect on D2 expression at cellular levels in cardiac FAPs.

Given the expression of D2 in the Tabula Muris, which indicate elevated D2 levels present in some fibroblasts and adipose cells from VAT, we next evaluated D2 expression in freshly isolated fibroblasts from mice expressing the D2-GFP under the PDGFR promoter.

This mouse model is necessary since it allows to specifically isolate cardiac fibroblasts by FACS procedure exploiting the GFP fluorescence marker expressed by these cells in this mouse strain.

Between the different cells found in the heart, FAP (Fibro-Adipogenic Progenitors) represent a common precursor of fibroblasts and adipose cells. We isolated FAP (Fibro-Adipogenic Progenitors) cells by FACS sorting from cardiac muscles of in PDGFr-eGFP mice. Cardiac FAPs were cultured in vitro and treated with 2μM of Vandetanib for 12 hours.

Also in these cells, Vandetanib caused a slight but significant decrease of D2 expression.

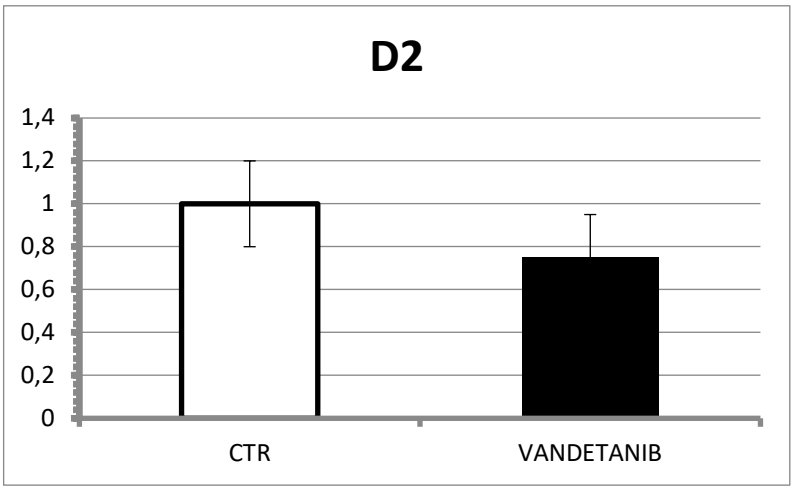

Figure 10: **D2 expression in FAP cell isolated from lower limb muscle and treated with Vandetanib.** D2 mRNA is slightly lower in treated cells p<0,1.

3.3 Effects of Vandetanib treatment on cardiac and systemic thyroid hormone target genes

Considering that D2 is the main enzyme causing increased T3 concentration at plasma and tissue levels, we evaluated the effect of Vandetanib on systemic and cardiac thyroid status. To this aim, we measured expression levels of well known thyroid hormone target genes: MHC1 as paradigm of a negative TH-target gene in the heart (Figure 11) and D1 as a positive TH-target gene in liver (Figure 12). Notably, vCMHC1, encoding for the MHC1 protein, was expressed at higher levels in heart in vandetanib-treated mice when compared to control mice; instead D1 concentrations was lower in liver of treated mice. These data suggested that Vandetanib treatment induces a tissue specific hypothyroid conditions, likely by affecting D2 levels.

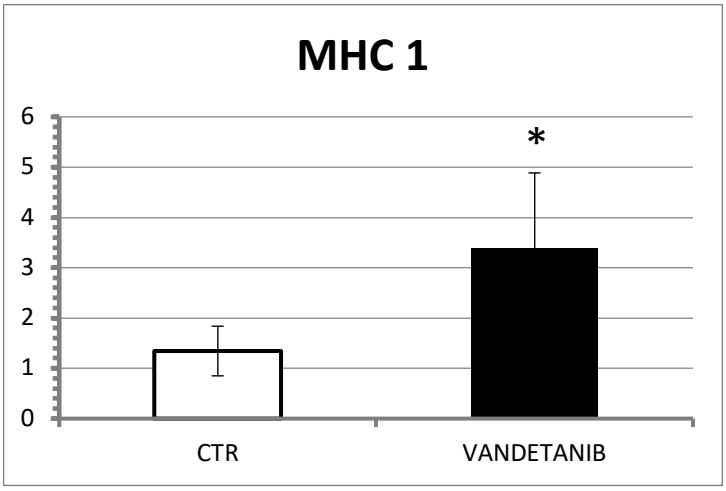

Figure 11: **MHC1 levels in heart of treated mice with Vandetanib.**
D2 mRNA is higher expressed in heart of treated mice. Data is significant * $p<0,03$

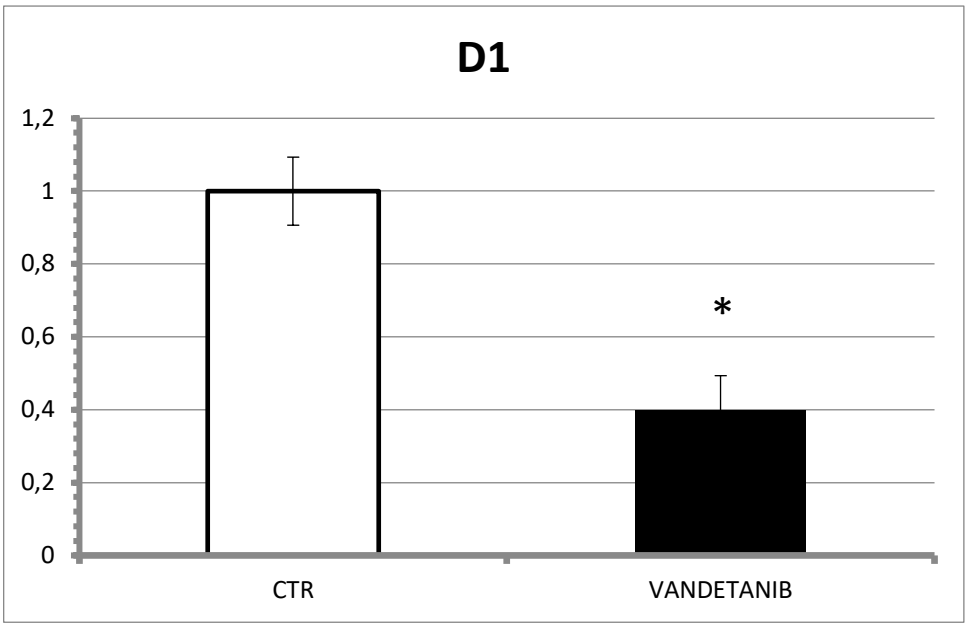

Figure 12: **D1 expression in liver of mice treated with Vandetanib.**
D1 mRNA is lower in mice treated with Vandetanib

3.3.1 Molecular mechanisms by which Vandetanib affects D2 expression levels.

To evaluate the molecular mechanisms by which Vandetanib affects D2 expression levels, we treated Human Embryonic Kidney 293 cells (HEK293) with increasing concentrations of Vandetanib (0.3µM - 1µM - 3µM) and we analyzed :

1) the functional activity of the thyroid hormone reporter plasmid T3tk-Luc;
2) the activity of D2-promoter through the reporter plasmid pD2-Luc.

Our results indicate that in the HEK293 T3tk-Luc activity as read-out of thyroid status was downregulated in cells treated with Vandetanib (Figure 13 A) and that 2) D2 promoter is less active upon Vandetanib treatment (Figure 13 B).
To evaluate if the Vandetanib treatment affect D2 protein levels also thought posttranscriptional mechanism we transfect a flagged D2 expression plasmid in HEK293 cells and we treat these cells for 48 h with Vandetanib. Protein extracts show that D2 protein levels is decreased if compared to untreated cells (Figure 13 C).
These experiments confirmed that Vandetanib induces a hypothyroid status in cells.

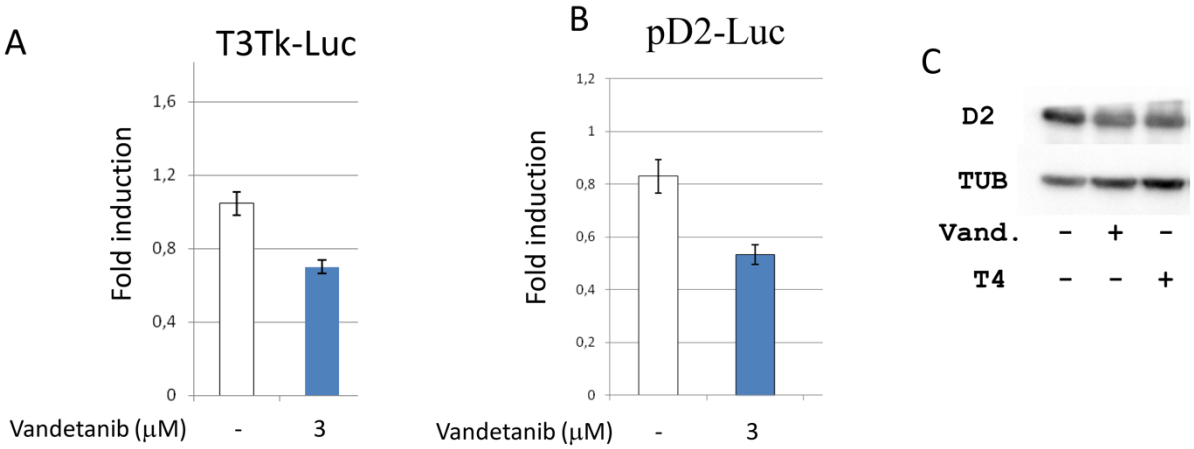

Figure 13: **Thyroid status, D2 expression and western blot of D2 in HEK293 treated with Vandetanib.**

A Thyroid status is downregulated in treated cells

B D2 expression is downregulated in treated cells

C D2 protein levels is decreased in treated cells

4 Discussion

TKIs represent an important therapeutic option for the treatment of radioiodine refractory (RAI-R) DTC, MTC and possibly for cases of poorly differentiated (PDTC) and anaplastic thyroid cancers (ATC). Hypothyroidism is a common side effect during TKIs cancer treatment (23). It occurs in patients that underwent total thyroidectomy for thyroid cancer as well as in patients treated with TKIs for other cancers and that not underwent thyroidectomy. Presently, not much evidences are present in the literature regarding the use of TKIs and peripheral hypothyroidism.

Our studies have showed that TKIs treatment causes systemic and peripheral hypothyroidism by modulating deiodinase homeostasis. Specifically, we provided evidence that D2 expression and activity are downregulated upon Vandetanib treatment.

Besides systemic regulation, the TH concentration in target tissues can differ remarkably depending on local TH metabolism, which ultimately regulates target gene expression. D2 enzyme catalyses the removal of an outer ring iodine atom from the pro-hormone T4 to generate the physiologically active product T3. The reduction of D2 causes hypothyroidism at a cellular level.

Several studies indicated that D2 is expressed in human and mouse heart, especially in the fibroblasts cell component (66); here we described for the first time the specific regions and cell compartment where D2 is located in the heart. In fact, using a novel generated mouse model, D2CRE^{ert}; Rosa26zsGreen, that allows to specifically track and visualize D2 expressing cells, we provided the first *in vivo* genetic evidence that D2 expressing cells are located in specific and restricted cardiac regions i.e. VAT, atrioventricular node and cardiac valve. This represents an important finding to explain the TKI-induced cardiac hypothyroidism.

To investigate whether Vandetanib affects D2 expression, we administered Vandetanib *in vivo* in 3 month old mice and we observed that D2 mRNA was downregulated in the heart of treated mice compared with control. The reduction in D2 levels causes tissue hypothyroidism as demonstrated by the increased expression levels of MHC1 and

decreased expression levels of D1. In cardiac hypothyroidism, heart rate and contractility are reduced, and peripheral vascular resistance is increased, leading to a reduction in cardiac output (43) and this is consistent with the ECG alterations observed under TKIs treatment (23).

Thyroid hormones have both direct and indirect effects on cardiac function. These effects are mediated through genomic and nongenomic mechanisms. T3 binds to a specific nuclear receptor which regulates the transcription of various genes with important roles in cardiovascular function (35). In fact, several studies have showed that thyroid hormones upregulate the expression of genes encoding sodium/potassium-transporting ATPases, increase the transcription of myosin heavy chain (MHC) α gene, and decrease that of MHC β gene, resulting in an increased velocity of contraction (33-36). Nowadays, no evidence exist in literature regarding the use of TKIs and thyroid hormone modulation on target gene. We have observed the effect of thyroid hormones on target gene at the cardiac level, in particularly on MHC1 as paradigm of a negative TH-target gene. Notably, we have observed that MHC1 levels in heart were higher in treated mice. Moreover, we have analyzed D1 concentrations as a positive TH-target gene for liver and we have observed that D1 levels were lower.

To demonstrate that FAPs are the cell target for Vandetanib in the heart, we evaluated D2 expression in freshly isolated FAPs from mice heart and also in these cells, Vandetanib caused a slight- but significant- decrease in D2 expression. This experiment also showed that of Vandetanib behaves *in vitro* as *in vivo*.

Here, we provide the first evidence that Vandetanib treatment induces a tissue specific hypothyroid status. In fact, our data demonstrate that Vandetanib downregulate D2 expression is peripheric and not only central and we have shown how this modulation is tissue specific.

Furthermore, we demonstrate that Vandetanib treatment affects D2 protein levels not only regulating mRNA levels but also thought post-transcriptional mechanism. Indeed, transfected D2 protein levels are decreased in Vandetanib treated HEK293 cells if

compared to untreated cells. To our knowledge, this represents the first observation of strong link between TKIs, e.g. Vandetanib, and D2 regulation.

We have demonstrated that D2 is downregulated by Vandetanib by in vivo and in vitro studies and this inhibition causes hypothyroidism, according to what is observed in patients undergoing therapy with Vandetanib. This is consistent with the need to increase the dose of levothyroxine in patients treated with TKIs for non-thyroid metastatic cancers and who have not undergone thyroidectomy.

Our findings suggest that D2 modulation has a critical role in Vandetanib induced hypothyroidism. Consistent with these results, regulation of deiodinases plays crucial roles in in the occurrence of TKIs induced adverse events.

Particularly at cardiac level, hypothyroidism causes changes in the expression of the target genes that induce cardiac dysfunction. Such a speculation might have important clinical relevance, since these alterations could influence discontinuation of treatment with TKIs or need dosage reduction.

Although these findings provide the first evidence that Vandetanib downregulated D2, yet a key question remains open: it is possible to identify patients who are most susceptible to developing clinically relevant cardiac abnormalities? Other studies are needed to address this issue.

In conclusion, our findings identify the Vandetanib downregulation of D2 enzyme as a cause of hypothyroidism and demonstrate that hypothyroidism induced by TKI is peripheral and tissue specific.

5 Materials and Methods

5.1 Animals

Animals were maintained in the animal facility at CEINGE Biotecnologie Avanzate, Naples, Italy. All mice used for experiments were adults, 8-12 week old. Animals were genotyped by PCR using tail DNA. C57BL/6 and PDGFr-eGFP were purchased from The Jackson Laboratory (Stock No: 000664 and 007669 respectively). D2CREert and Rosa26zsGreen were kindly provided by Prof. Douglas Forrest (National Institutes of Health (NIH), United States).

5.2 Cell isolation, culture and transfection

To isolate FAP, mouse heart was digested in PBS containing 1mg/ml Dispase II (Roche), 1mg/ml collagenase A (Sigma Aldrich) and 2.5 mM CaCl2 for 30 minutes at 37°C. Following the digestion, the tissue suspension was filtered in 70 μm cell strainers (Corning) and centrifugated at 600g for 15 min at 4°C; the pellet was resuspended in cold DMEM and 1% penicillin-streptomycin supplemented with 2% FBS, and the cell suspension was filtered through a 30 μm strainer (BD Bioscience). FACS was performed using FACS Aria IIIu (Becton Dickinson).

HEK293 cells obtained from ATCC and FAP were cultured in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% FBS (Microgem), 2 mM glutamine, 50 i.u. penicillin, and 50 μg/ml streptomycin. Cells were treated with different concentration as indicated of Thyroid Hormone and Vandetanib. All transient transfections were performed using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions.

5.3 Tissue preparation and analysis

Histology, Immunostaining and Immunoblotting. Mouse tissue was fixed in 4% PFA and embedded in paraffin wax, 6 um serial paraffin sections of mouse heart was cut and stained with DAPI; the acquisition were executed using the OLYMPUS IX51 microscope and CellF Software.

5.4 RNA extraction and RT-PCR analysis

mRNAs were extracted with TRIzol reagent (Life Technologies Ltd). cDNAs were prepared with SuperScript VILO Master Mix (Life Technologies Ltd) as indicated by the manufacturer. The cDNAs were amplified by PCR in an iQ5 Multicolor Real-Time Detector System (Bio-Rad) with the fluorescent double-stranded DNA-binding dye SYBR Green (Applied Biosystems). Specific primers for each gene were designed to work under the same cycling conditions (95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min), generating products of comparable sizes (~200 bp for each amplification). Primer combinations were positioned whenever possible to span an exon-exon junction and the genomic DNA contamination were removed with deoxyribonuclease. All samples were run in triplicate. The template concentration was calculated from the cycle number when the amount of PCR product passed a threshold established in the exponential phase of the PCR. The relative amounts of gene expression were normalized using cyclophilin A expression as an internal standard (calibrator). The results, expressed as n-fold differences in target gene expression, were determined as follows: $n \times \text{target} = 2^{(\Delta Ct\text{sample} - \Delta Ct\text{calibrator})}$.

5.5 Luciferase assay

The luciferase reporter plasmids TRE3-TkLuc or the pTRE3G-Luc and CMV-Renilla were co-transfected into cells. Luc activities were measured 48 hours after transfection with the Dual Luciferase Reporter Assay System (Promega, Madison, Wisconsin, USA), and differences in transfection efficiency were corrected relative to the level of Renilla Luciferase. Each construct was studied in triplicate in at least three separate transfection experiments.

5.6 Protein exctraction and Western blot

Transfected HEK293 cells were lysed in buffer containing: 0.125 M Tris pH 8.6; 3% SDS, protease inhibitors including PMSF 1 mM and phosphatase inhibitors. Total protein extracts were run on a 10% SDS-PAGE gel and transferred onto an Immobilon-P transfer membrane (Millipore). The membrane was then blocked with 5% non-fat dry milk in PBS, 0.2% Tween. The anti-FlagM2 and anti-Tubulin primary antibodies (Sigma Aldrich) were used respectively 1:1000 and 1:5000 in PBS, 0.2% Tween, 5% non-fat dry milk. Anti-mouse IgG-horseradish peroxidase (HRP) secondary antibodies (BIORAD) were detected by chemiluminescence (Millipore, cat. WBKLS0500). Anti-Tubulin specific antibodies (Sigma Aldrich) were used as loading control. All Western blots were run in triplicate, and bands were quantified with ImageJ software.

5.7 Statistical analysis

Results are presented as means_S.D. Comparisons between two groups were performed using Student's *t*-test or ANOVA when there were three or more groups simultaneously involved. Duncan's test was used for multiple comparisons. All densitometry data were rank transformed prior to analysis. $P<0{\cdot}05$ was considered statistically significant.

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