Clinical and Molecular Studies on Differentiated Thyroid Carcinoma Management

Randa M Abdulrahman Hareedy

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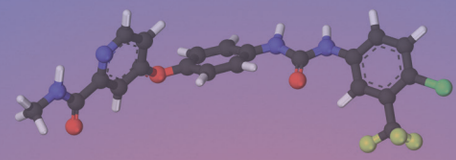

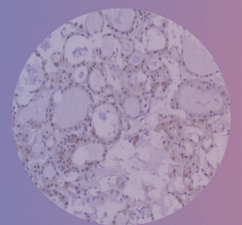

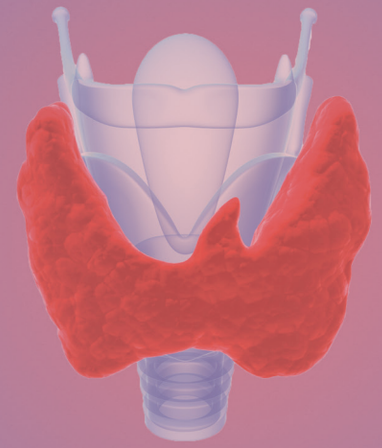

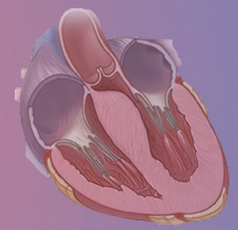

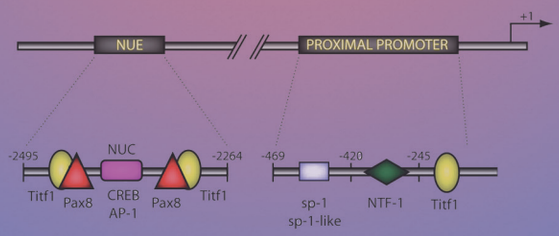

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"It was the best of times, it was the worst of times, it was the age of wisdom, it was the age of foolishness, it was the epoch of belief, it was the epoch of incredulity, it was the season of Light, it was the season of Darkness, it was the spring of hope, it was the winter of despair, we had everything before us, we had nothing before us, we were all going direct to heaven, we were all going direct the other way - in short, the period was so far like the present period, for good or for evil, in the superlative degree of comparison only."

Charles Dickens *(A Tale of two cities; 1859)*

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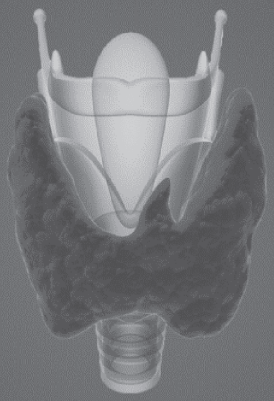
CHAPTER 1

General Introduction



INTRODUCTION

Thyroid carcinoma is the most common endocrine malignancy accounting for >90% of malignancies of endocrine glands (1), but the overall incidence is low. Differentiated thyroid carcinoma (DTC), which is the most prevalent thyroid carcinoma, has an excellent 10-year prognosis of around 85%, which is due to the biological behavior of DTC (2, 3) and the available treatment options (4). Effective treatment in most DTC patients consists of total thyroidectomy followed by therapy with radioactive iodide (RAI), which is based on the unique ability of follicular thyroid cells to take up iodine via the Sodium Iodide Symporter (NIS) (5).

Despite the excellent prognosis of DTC, clinical challenges remain. First, the diagnosis of DTC is hampered by the fact that although the incidence is low, thyroid nodules are prevalent. Effective diagnostic strategies are warranted to identify patients with clinically relevant DTC without subjecting the many patients with thyroid nodules to unnecessary and invasive procedures. In this thesis, the diagnostic value of a potential marker for DTC is being evaluated. Although prognosis of DTC is favorable, unfortunately, in a high proportion of patients with metastases the capacity for RAI accumulation is diminished or lost. In this thesis, fundamental aspects of the regulation of NIS are being studied, that may have implications for improving RAI therapy. Furthermore, the long-term efficacy of a new treatment with the multikinase inhibitor sorafenib is evaluated. As this treatment is associated with various adverse events, including hypothyroidism, a potential mechanism of sorafenib associated hypothyroidism is evaluated. Finally, DTC patients have traditionally been treated with high dosages of levothyroxine substitution, in order to suppress serum thyroid stimulating hormone (TSH) levels. However, high thyroid hormone concentrations may have adverse effects on various organ systems, including the heart. In this thesis, we evaluate the effects of various thyroid hormone concentrations on cardiac function in DTC, using dedicated cardiac ultrasound.

THYROID PHYSIOLOGY

The cornerstone of the non-surgical therapy of DTC is the ability of the thyroid gland to accumulate iodide through NIS. NIS is a glycoprotein located at the basolateral plasma membrane of thyrocytes. It actively accumulates iodide by a factor of 20 to 40 relative to its concentration in the blood. Although other tissues such as breast, stomach and salivary gland have been reported to accumulate iodide, the thyroid gland is able to retain iodide due to its

ability to oxidize iodide by thyroid peroxidase (TPO) and its subsequent organification into thyroglobulin (Tg) (6). NIS has a long half-life of about 7 days with a complex transcriptional, post-transcriptional, and post-translational regulation in order to target NIS to the plasma membrane; the only location where NIS is functional. TSH stimulates NIS transcription and *de novo* synthesis, and plays a role in the targeting of NIS to the plasma membrane (6).

After uptake of iodide into the thyroid epithelial cells, it is subsequently transported to the follicular lumen by pendrin (6). At the apical plasma membrane, iodide is oxidized by TPO and coupled to tyrosine residues in Tg, forming monoiodotyrosines (MIT) and diiodotyrosines (DIT). The next step in thyroid hormone synthesis is the coupling of two iodotyrosyl residues (6). Iodinated Tg is subsequently endocytosed and hydrolyzed in the lysosomes thus releasing thyroxine (T4) and triiodothyronine (T3). The synthesis of thyroid hormones is regulated by the hypothalamus-pituitary-thyroid axis. Thyrotropin releasing hormone (TRH), which is produced by the hypothalamus, stimulates the secretion of TSH by the anterior pituitary.

T3 is the active form of thyroid hormone. Circulating T3 produced by the thyroid gland accounts for only 20% of T3 (7). The majority of T3 is formed after conversion of T4 into T3 in peripheral tissues like liver, kidney and muscle. T4 and T3 exert a negative feedback on TRH and TSH secretion.

Peripheral thyroid metabolism is mainly regulated by the iodothyronine deiodinases D1, D2, and D3 (8). D1 is present in the thyroid, liver and kidneys and converts T4 to T3. In addition, it plays a role in the breakdown of reverse T3 (9). D2 enhances local T3 production in various tissues. It is expressed in brain, skeletal muscle, thyroid, pituitary, brown adipose tissue, aortic smooth muscle cells, osteoblasts and the heart (8, 10). In the brain, D2 is expressed mainly in astrocytes in the cerebral cortex, hippocampus and amygdala (8). D3 inactivates T3 and T4 and thus regulates the clearance of T3 and T4. It is present in the brain, skin, placenta and fetal tissues.

Thyroid hormone has numerous effects on growth, differentiation and metabolism (11). Thyroid hormone receptors (TR) that can be distinguished in TRα and TRβ are nuclear receptors that bind to specific DNA binding sites, called thyroid hormone responsive elements (TRE). Binding of T3 to TR can either lead to transcription or repression of T3 dependent genes. Activation or repression of TRE is a complex process in which co-activators and repressors play a role. In addition, TR can form homo- or heterodimers (12, 13). Interestingly,

non-genomic actions of thyroid hormones through non-nuclear TR have been postulated (14-19).

DIFFERENTIATED THYROID CARCINOMA

Epidemiology

Although thyroid nodules are common, more than 95% of these nodules are benign (20) thyroid cancers represent 1.7% of newly discovered cancer cases in the world according to GLOBOCAN (2002-2008) with a female to male ratio 2.3:1 (21). In Europe, thyroid cancer has the highest incidence and prevalence of endocrine carcinomas (1). Worldwide, the incidence of thyroid carcinoma is increasing. In the Netherlands the annual overall incidence of thyroid cancers has risen from 2.0 in 1989 to 3.25 per 100.000 person/years in 2011 with a female to male ratio of 2.4:1 (22) accompanied with an increase in the 5-years survival rate from ~76% in 1993 to 82% in 2011. The increase in incidence can be explained by the more intensive use of imaging modalities that increased the detection of small, non-symptomatic tumors (23).

Thyroid cancer pathology

Thyroid carcinomas are categorized into 3 main groups. DTC (97%) and anaplastic thyroid carcinoma (ATC) (1%) originate from follicular epithelial cells, whereas medullary thyroid carcinoma (2%) originates from neuroendocrine parafollicular cells. DTC can be distinguished in many histological subtypes, major groups being papillary thyroid carcinoma (PTC), which is the most common type of thyroid cancers (87% of thyroid carcinomas), and follicular thyroid carcinoma (6%). Hürthle cell carcinomas (3-4%) form a distinct subgroup of DTC that are mostly FTC but can also be PTC. Mixed FTC and PTC also occur. The follicular variant of PTC (FVPTC) is a subtype of PTC that is hard to differentiate from benign follicular adenoma and is indeed considered by some as a benign tumor.

Molecular pathogenesis

The genetic events involved in DTC include gene rearrangements and mutations. As a consequence of these genetic mutations and/or rearrangements, signaling pathways are activated in favor of cell growth, survival and angiogenesis. PTC frequently harbor point mutations of BRAF (30-69%) and rearrangements of the RET oncogene (40-70%) leading to MAP-kinase pathway activation (24-27). To a lesser extent, RAS gene mutations also occur in PTC (10-20%). FTC frequently harbors RAS mutations (18-52%) and/or PPARγ/PAX8

rearrangements (25-56%) (28). Mutations of PTEN may also be present in FTC. RAS mutations may activate both MAPK and PI3K/AKT signaling pathways. Genetic alterations involving the PI3K/AKT signaling pathway are rare in DTC.

In ATC, genetic alterations in the PI3K/AKT signaling pathway may be present (29-31). Also, mutations of the p53 tumor suppressor gene (32) are involved. Recently mutations in the β-catenin gene have been described in ATC (33-35).

Dedifferentiation

DTC initially retains the biological properties (iodide uptake, Tg synthesis and response to TSH) of normal thyroid cells (36-38). However, NIS, TPO, Tg and TSH receptor (TSHR) expression gradually decrease in DTC with NIS and TPO disappearing at an earlier stage than other proteins (39-41). NIS expression has been studied extensively. Most studies showed reduction or even absence of NIS mRNA in thyroid carcinomas (42-44), while immunohistochemistry studies have demonstrated that NIS protein is over-expressed in many thyroid tumors and is predominantly located in the cytoplasm (36, 40). TSHR expression is persistent in nearly all DTC (40, 45, 46). Tg production remains preserved even in the absence of iodide uptake. In ATC, Tg secretion may decrease and is eventually lost (40, 45, 46). A causal relationship between genetic alterations and loss of functional NIS expression is present: tumor cells harboring BRAFV600E mutation have decreased NIS, TPO and TSHR gene expression compared to other tumor cells without this mutation (47-51). In addition, a relationship between activation of PI3K and mTOR and loss of NIS expression has also been described (52).

Diagnosis

Ultrasonography (US) guided fine needle aspiration cytology (FNAC) is the cornerstone of the evaluation of thyroid nodules for malignancy (53). Diagnostic accuracy of FNAC for DTC is influenced by decision rules for subsequent thyroid surgery. Sensitivity of FNA for DTC is generally regarded as 90-95%. The specificity is lower (60-80%) when all patients with a non-benign FNA are referred for surgery (54). FNAC results are currently described according to the Bethesda classification (see table 1) (53). The problem is that with the increased application of US, more small asymptomatic nodules are being discovered, that even if they harbor DTC, may not have clinical consequences at all. Yet, when DTC is diagnosed by FNA, most patients will undergo surgery, which explains the impressive rise in DTC incidence. A rational selection of patients who are candidates for FNA is thus warranted, and should be

mainly directed by clinical suspicion and/or US selection by experienced radiologists as recommended in the updated Dutch thyroid carcinoma guidelines (www.oncoline.nl).

Indeterminate FNAC results (Bethesda class III and IV) constitute 17-30% of cases with an overall risk of malignancy of up to 30% (55). Bethesda class III and IV may correspond with FVPTC, FTC or FA (56). Most of these indeterminate cases are referred for diagnostic hemithyroidectomy, but the majority of patients will have benign disease. Yet they are exposed to a 2-10% risk of surgical complications, and most patients will require levothyroxine replacement therapy for life (57). Therefore improved preoperative diagnostic evaluation for patients with indeterminate cytologic findings on FNA is warranted. In order to improve the preoperative diagnostic evaluation for patients with indeterminate cytologic findings on FNA, additional approaches have emerged to improve the accuracy of the diagnosis, most importantly immunohistochemical and molecular genetic markers.

Table1: FNAC classification with Bethesda classification terminology

Class	Cytology result	Frequency (%)	PPV (malignancy)	Management
I	Non-Diagnostic/ Unsatisfactory	2- 20	4	Repeat FNAC
II	Benign	60- 70	3	Clinical follow-up
III	Atypia of Undetermined Significance/ Follicular Lesions of Undetermined significance	3- 15	5- 15 (Estimated)	Repeat FNA
IV	Follicular Neoplasm/ Suspicious of Follicular Neoplasm (Hurthle included)	15- 30	15- 30	Surgical intervention
V	Suspicious of Malignancy	2- 3	60- 75	Surgical intervention
VI	Malignancy	3- 7	97- 99	Surgical intervention

Data adapted from (Cibas and Ali, AmJClinPathol, 2009(58) and Baloch et al, Diagnostic cytopathology, 2008 (59). PPV (malignancy): positive predictive value for malignancy

Immunohistochemical markers

Microarray technology enabled molecular characterization of thyroid pathologies and biomarkers to classify indeterminate thyroid follicular lesions. Galectin-3, HBME-1, and

CK19 have been identified as the most promising makers so far (60). HBME-1 is considered the most sensitive marker for PTC and was even found to be useful in the differentiation between FA and FVPTC (61, 62). The problem with these markers is that their sensitivity progressively declines for the diagnosis and differentiation of FVPTC, FTC, and less common malignant variants (63).

Bone morphogeneic protein 7 (BMP7) is an interesting protein to study in the context of DTC as BMP7 among other bone morphogenic proteins may be involved in thyroid proliferation (64, 65). Unlike the oncogenic actions of TGF-β (also a member of the same superfamily) in tumorigenesis, BMP7 appears to be involved in the preservation of the epithelial phenotype (66); and showed anti-tumor effect in breast, prostate cancers and anaplastic thyroid carcinoma cell lines (67). Using tissue microarray (TMA), which facilitates a comprehensive expression analysis of proteins in multiple tissues (68, 69) we studied the differential expression of the proteins in the BMP7 signaling pathway as a possible diagnostic biomarker for thyroid lesions (**Chapter 2**).

Molecular diagnostics

Since 60 to 70% of thyroid cancers harbor at least one known genetic mutation, molecular analysis of thyroid tissue is a promising strategy to improve diagnosis in the indeterminate group of FNAC. The presence of established genetic alterations in FNA indicates malignancy with high specificity (70), which can lead to a recommendation for total thyroidectomy rather than for hemithyroidectomy. This approach is in particular useful when the pretest likelihood for malignancy is high (71).

Another clinical application of molecular genetics in thyroid cancer is represented by the Gene Expression Classifier (GEC) (Veracyte, Inc.; South San Francisco, CA). GEC is a 167-gene expression assay used to further re-diagnose cytological indeterminate samples into either benign or suspicious for malignancy. The ability of GEC to reclassify Bethesda III and IV nodules as benign or non-benign has a negative predictive value of > 94% implying that thyroid nodules with indeterminate cytologic abnormalities and benign gene-expression classifier results have a post-test probability of malignancy that is similar to the probability for nodules with cytologically benign features on FNAC (72).

Initial therapy

According to the consensus of the European Thyroid Association (ETA) and the guidelines of the American Thyroid Association (ATA) (73, 74), most patients with DTC should be

subjected to initial therapy consisting of near-total thyroidectomy followed by RAI remnant ablation therapy. Very-low-risk patients (small unifocal intrathyroidal lesions) may be treated with hemithyroidectomy alone. When lymph node metastases are present, modified neck dissection of the affected side is recommended, rather than picking-out.

In recent years, the benefits of RAI ablation therapy are debated and a strategy based on risk of recurrence is recommended (73). RAI ablation is considered beneficial in high-risk patients (incomplete resection, distant metastases, lymph node metastases, aggressive histologies). For other patients, the effects of RAI on recurrence are controversial. As RAI is associated with side effects and a small but significant risk for other malignancies (75), the current position is to limit the indications for RAI and to use lower dosages. Recent landmark studies from France (ESTAMIBL) and the UK (HiLo) have indicated that 37 mCi is as efficacious as 100 mCi for thyroid remnant ablation (76, 77). Studies in the UK and France are now underway to evaluate abstention from RAI in intermediate risk patient groups.

Successful RAI therapy is dependent on the stimulation of iodide uptake by TSH. In terms of preparation for RAI, high TSH levels can be realized either by discontinuation of levothyroxine therapy or by the application of rhTSH during levothyroxine therapy. In the ESTAMIBL and HiLo studies, ablation success was comparable between levothyroxine withdrawal and rhTSH. Withdrawal however is poorly tolerated by patients and negatively affects quality of their life (78). The ultimate conclusion on comparability between thyroid hormone withdrawal and rhTSH can be drawn when long-term results on clinical outcome between the 2 approaches become available.

The assumption of rhTSH aided RAI therapy is that the continuation of levothyroxine substitution has no effect on iodide uptake, or, in other words, that T3 does not have a direct effect on NIS expression. To investigate this important question, in **Chapter 3**, we performed in vitro studies in thyroid cell lines, to verify a direct effect of T3 on NIS expression and iodide uptake.

Evaluation of initial therapy and follow-up

The aim of the evaluation of initial therapy is to restratify patients in groups who are likely to be cured and groups with persistent disease or high risk for recurrent disease. As most patients will be cured after initial therapy, this restratification will allow a limited follow-up protocol in the first group and an intense follow-up program in the latter group.

Ultrasound

An essential element in the evaluation of initial therapy is ultrasound of the neck, which is performed 6-9 months after surgery. Ultrasound combined with FNA has the highest sensitivity (94%) for local recurrence and lymph node metastases (79, 80) and thus, ultrasound has an important role in the follow-up of DTC. When suspicious lesions are present, FNA will be performed and when tumor is present, the patient will undergo re-surgery, when cure is possible.

Thyroglobulin

The cornerstone in the biochemical evaluation of primary therapy are measurements of serum Tg. Serum Tg should be below an institutionally defined cut off level after total thyroidectomy and postoperative RAI ablation therapy. Until recently, it was recommended to measure Tg during TSH stimulation (73, 74). As in RAI ablation, traditionally, this approach was achieved by levothyroxine withdrawal. Currently, exogenous elevation of TSH levels is achieved via rhTSH injections without subjecting the patients to the disadvantages of iatrogenic hypothyroidism. The diagnostic sensitivity and negative predictive value of combining rhTSH stimulated Tg measurements with neck ultrasound is 96.3% and 99.5% respectively (81, 82). The advent of high sensitive Tg measurements has revealed that unstimulated Tg measurements have comparable diagnostic accuracy as rhTSH stimulated Tg measurements in subgroups of DTC patients with either very low or high Tg levels (83).

When antibodies against Tg (TgAb) are present, Tg measurements are considered less reliable, and other imaging techniques (ultrasound and others) are needed to establish cure. Although TgAb titers may be related to disease state in epidemiological studies, they have insufficient diagnostic accuracy for use as tumor marker in individual patients (84).

Diagnostic whole body scan (WBS)

Diagnostic whole body scan (WBS) with RAI is no longer recommended for routine evaluation of the success of initial therapy in all patients and is now only recommended when TgAb are present or in selected subgroups of high risk patients (74).

Restratification

According to retrospective studies of Castagna and Tuttle, DTC patients with undetectable Tg levels, absent TgAb and negative ultrasound after initial therapy have a very low risk of recurrence and death and could be followed by yearly unstimulated Tg measurements.

Patients with elevated Tg levels may be followed by repeat Tg measurements, as Tg levels may decrease in a substantial proportion of patients during the first year after initial therapy (85, 86). When Tg levels increase, imaging (CT and/or FDG-PET) may be performed and RAI therapy may be performed. Presence of FDG-PET positive lesions is related with a worse prognosis (87, 88).

Patients with high-risk of recurrence and metastasis are followed up with regular Tg measurements and/or imaging procedures.

TSH suppression

Until recently, it was recommended in all DTC patients, irrespective of their disease state, to maintain low TSH levels, as TSH is considered as a tumor promoting hormone (37). As recent studies have demonstrated a negative effect of TSH on prognosis only in high-risk patients (88, 89), it is now recommended to maintain low TSH levels (<0.1 mU/L) in high-risk patients or patients with active disease while in others, levothyroxine therapy can be titrated to normal TSH levels (20, 37).

Recurrent or persistent disease

Conventional therapy

When a malignant lesion is accessible surgery can be performed. In the majority of patients, this will be loco-regional recurrences with no distant metastases. In solitary pulmonary, bone or brain metastases, surgery can also be attempted. If a malignant lesion is present which cannot be cured by surgery, RAI therapy may be performed. The remission rate in pulmonary metastases treated with RAI varies from 90% in patients with microscopic metastases to 10% in macroscopic metastatic disease and from 20-7% in bone metastases (90, 91). High cumulative dosages of RAI are associated with increased risk of secondary malignancies in addition to other complications (92-94).

Palliative therapies include external irradiation, radiofrequency ablation, chemoembolization and for pulmonary metastases, endobronchial laser therapy (95-98).

Resistance to radioactive iodide

Five to 15% of DTC patients will develop or present with local recurrent disease or metastasis. In 25-66% of these patients, the susceptibility to RAI is diminished or lost (4). The clinical definition of RAI refractoriness that also was used in the DECISION trial (see below) includes the absence of RAI accumulation in one or more lesions on whole body scintigraphy

after RAI therapy and/or the absence of a beneficial effect of RAI, despite uptake of RAI. It has been established by a French group that no additional beneficial effect of RAI is observed after a cumulative dose of 600 mCi (3). Absence of a beneficial effect of RAI can be defined as disease progression, according to RECIST, within 1 year after the last RAI therapy (99).

The mechanism of RAI refractoriness not only involves the loss of NIS expression, which is related with genetic changes in DTC (see earlier), but also defective transport of NIS to the cell membrane. Furthermore, changes in RAI kinetics, related to the loss of follicular architecture and/or defects in the apoptotic machinery in DTC may be involved.

Treatment options for patients with RAI resistance were until recently limited to palliative treatment, as conventional chemotherapy is not effective in DTC. Recently, novel treatment options have become available in terms of kinase inhibitors.

KINASE INHIBITORS

Kinase inhibitors selectively bind to the substrate binding domain of intracellular signaling peptides with kinase activity. In many types of cancer, genetic alterations directly or indirectly lead to the activation of kinase pathways. Therefore, the identification of activated kinase pathways as well as the development of compounds that selectively interfere with these pathways has offered important new perspectives for cancer therapy. The identification of the molecular pathogenesis of DTC (see earlier) has led to the selection of kinase inhibitors that specifically interfere with these pathways and therefore open new roads in the treatment of RAI refractory DTC. Kinase inhibitors target either one specific kinase (selective KI) or multiple kinases (MKI) (100, 101).

Motesanib was the first MKI to be tested in a multicenter, open-label phase II trial in 93 patients with radiographically progressive DTC resulting in a partial response (PR) of 14% according to RECIST criteria (102, 103). Other MKIs have been studied for their efficacy and safety in phase II trials, such as axitinib (104), sunitinib (105), imatinib (106), cabozantinib (107), lenvatinib (108) and sorafenib (109-121) (table 2).

Some MKI have been investigated in phase III studies. The DECISION trial, with sorafenib, was the first phase III study with a MKI in DTC (121). A phase III study with lenvatinib (SELECT study) was recently completed (126). Data of DECISION and SELECT are discussed below. Vandetinib is currently being studied in the phase III VERIFY study (127).

In addition to MKI, compounds have been developed that selectively target mutated kinases. Vemurafenib, which specifically targets mutated BRAFV600E, has been studied in patients with BRAFV600E positive PTC. Median progression free survival (PFS) after treatment with vemurafenib in treatment naive patients was 15.6 months, in patients who failed on sorafenib 6.8 months (128). Another recent study involved 15 BRAFV600E positive PTC patients, where 47% showed PR (129).

Table 2: Clinical trials of Tyrosine Kinase inhibitors in advanced thyroid cancer with focus on differentiated thyroid carcinoma (DTC)

Drug (category)	**Target**	**Study Phase**	**Clinical pathology**	**Patients (n)**	**Response**				**Ref.**
					PFS (mon)	**CR%**	**PR%**	**SD%**	
Sorafenib (MKI)	PDGFR RAF, RET VEGFR-1, 2	II	DTC MTC	34	NR		21	65	(109)
		II	DTC* MTC ATC	30	18 19.5*		23*	53*	(116)
		II	DTC	31	13.5		25	34	(110)
		II	DTC/PTC	41	15		15	23	(117)
		II	DTC* MTC ATC	34	13.5		19*	50*	(120)
		III	DTC vs placebo	417 (207 vs 210)	10.8 vs 5.8		12 vs 0.5		(121)
Lenvatinib (MKI)	VEGFR1,3 FGFR1-4 PDGFRβ RET, cKit	III	DTC vs Placebo	392	18.3 vs 3.6 (Median)	1.5 vs 0	63.2 vs 1.5		(126)
Motesanib (MKI)	VEGFR-1,2, 3 PDGFR, cKit	II	DTC	93	9.3		14	67	(102)

Continue Table 2: Clinical trials of Tyrosine Kinase inhibitors in advanced thyroid cancer with focus on differentiated thyroid carcinoma (DTC)

Axitinib (MKI)	VEGFR-1, 2, 3 PDGFR cKit	II	DTC MTC ATC	59	18	30	42	(104)
Sunitinib (MKI)	FLT3, KIT PDGFR	II	DTC MTC	35	3	28	46	(105)
	VEGFR-2	II	DTC MTC	43	12.8	13	68	(122)
Vandetanib (MKI)	VEGFR-2 EGFR	III	DTC vs placebo	72 vs 73	11.1 vs 5.9	43		(123)
Pazopanib (MKI)	PDGFR, VEGFR-1,2, 3& cKit	II	DTC	37		49		(124)
Selumetinib	MEK-1/2	II	PTC	32	7.4	3	54	(100)
(SKI)		II	PTC	20	New/ increase RAI uptake in 60% of patients (100% with NRAS mutations). PR 5/8; SD 3/8			(125)

MKI: multikinase inhibitor; SKI: single kinase inhibitor; PFS: mean Progression Free Survival; CR: complete recovery; PR: Partial response; SD: Stable Disease.

Sorafenib (BAY 43-9006) is an oral MKI targeting the serine/threonine kinase wild-type and mutant BRAF V600E, along with several other tyrosine-kinases including RET and the angiogenic tyrosine kinase growth receptors (VEGFR 2,VEGFR 3 and PDGFR beta). It also targets c-KIT and Flt-3 (109, 114). The drug actions in thyroid cancer are thus antiproliferative and anti-angiogenic.

Sorafenib has been approved by the the FDA and the EMA for the treatment of metastatic RAI resistant DTC (130, 131). Between 2008 and 2013 sorafenib was studied in a number of phase II clinical trials, one retrospective longitudinal study and 1 phase III, randomized, placebo-controlled, multicenter trial study (DECISION trial) (109, 110, 114, 116, 117, 119-121, 132). The phase II studies included either DTC patients only or DTC with ATC and MTC. The median PFS in phase II studies ranged from 13-19.5 months. PR rates observed in these studies ranged from 15%-25%. In **Chapter 4**, we report the long-term outcomes of one of these studies (110).

The DECISION trial is a multicentre, randomised, double-blind, placebo-controlled, phase 3 trial in which 417 patients with radioactive iodide-refractory locally advanced or metastatic DTC that had progressed within the past 14 months were treated with sorafenib (400 mg orally twice daily) or placebo. Median PFS was significantly longer in the sorafenib group (10.8 months) than in the placebo group (5.8 months). PFS improved in all prespecified clinical and genetic biomarker subgroups, irrespective of mutation status (121).

The SELECT study is randomized, double-blind, placebo -controlled phase III study in which 392 DTC patients with the same characteristics as DECISION were randomized 2:1 to lenvatinib or placebo. Median PFS was 18.3 months in lenvatinib vs. 3.6 months in placebo. Benefit was observed in all predefined subgroups (126)

Both studies have for the first time shown that MKI have a benefit on PFS in patients with advanced DTC and are therefore landmark studies. Questions to be answered remain the selection of subgroups of patients who benefit most from these drugs, the optimal time point of initiation of therapy and the optimal dosing strategy. It is now recommended to initiate therapy with MKI when RAI resistance is present, when progression has been established according to RECIST and when considerable tumor load is present (99) .

Toxicity is common in all kinase inhibitors. Most of the side effects include muco-cutaneous problems (hand foot syndrome, skin rash, stomatitis, dysphonia, photosensitivity, keratoacanthomas and malignant squamous cell lesion), fatigue, weight loss, cardiovascular manifestations (hypertension, QT interval prolongation and thromboembolism) and gastrointestinal manifestations (diarrhea and nausea). In the DECISION trial, adverse events occurred in 99% patients receiving sorafenib during the double-blind period and in 88% patients receiving placebo. Most adverse events were grade 1 or 2. The most frequent treatment-emergent adverse events in the sorafenib group were hand-foot skin reaction (76%), diarrhoea (68·6%), alopecia (67%), and rash or desquamation (50%) (121). In the SELECT study, the 5 most common adverse events of lenvatinib were hypertension (68%), diarrhea (59%), weight loss (46%), nausea (41%) and proteinuria (126).

Metabolic side effects are reported in MKI therapy, including hypocalcaemia and hypothyroidism (133, 134). Hypothyroidism has been reported in motesanib (102), sorafenib (135), imatinib (136, 137), sunitinib (135) and vandetanib requiring adjustments in the dosage of thyroid hormone replacement therapy (138). As DTC patients are athyreotic after surgery, hypothyroidism has to be related to either decreased absorption of levothyroxine or increased

degradation. In **chapter 5,** we studied the hypothesis that sorafenib enhances peripheral inactivation of thyroid hormone via increased activity of type 3 deiodinase.

REDIFFERENTIATION

Despite the fact that the above mentioned studies with MKI offer important new perspectives for DTC patients with RAI refractory tumors, the proportion of complete remissions is very low. Consequently, additional approaches are warranted, which include both combination therapies as well as strategies aimed at redifferentiation.

In recent years, a number of studies have been performed to investigate the potential to induce redifferentiation of thyroid carcinoma cells, with retinoic acid, lithium and histone modification agents (139-141). These treatments however had only a modest clinical benefit. Preclinical studies showed that switching off the BRAF activation, either genetically or through inhibition of its downstream signalling molecules (MAPK), restores the iodide uptake in TC cells and that selective MAPK pathway antagonists increase the iodide uptake in these cells (142-144). Based on these observations Ho et al carried out a trial in 20 patients with advanced, RAI refractory DTC who underwent treatment with selumetinib for 4 weeks after which RAI uptake was evaluated. Patients showing sufficient iodide uptake (8/20) were subsequently treated with RAI. Of these, 5 had confirmed PR and 3 had stable disease. Moreover enhanced iodide uptake was also found in bone and nodal metastases (125).

In **Chapter 6,** we performed in depth studies on the regulation of functional NIS expression, in order to identify new approaches to enhance RAI uptake in DTC. These studies are based on the observation that 5`Adenosine monophosphate-activated protein kinase (AMPK) plays an important role in NIS expression.

Both AMPK and thyroid hormone play a crucial role in energy metabolism (11, 145). Recently, AMPK has been associated with direct regulatory effects on thyroid function (146, 147). AMPK is an energy sensing heterotrimeric complex that plays an important role in the energy balance on both the cellular level and the whole body by balancing nutrient supply and demand (145).

Metformin is the first-line drug of choice for the treatment of type 2 diabetes (148). The AMPK-dependent signalling pathway is considered the key effector of the action of metformin (148, 149). Two recent studies have shown that AMPK activation in thyrocytes decreases iodide uptake and NIS expression and increases glucose uptake (146, 147). In **Chapter 6**, we examined the effect of AMPK modulators (metformin and CompoundC) on

thyrocyte function both in-vivo and in-vitro and the transcriptional regulatory role of the AMPK- dependent signalling pathway on NIS protein expression.

ADVERSE EFFECTS OF ABNORMAL THYROID HORMONE SERUM CONCENTRATIONS IN DTC PATIENTS

Patients with DTC experience abnormal thyroid hormone levels in two ways: in many patients, levothyroxine substitution is withdrawn in preparation for RAI therapy in order to realize high TSH levels. As a consequence, patients will experience episodes of hypothyroidism, which may have disadvantageous effects.

As mentioned above (*Evaluation of primary therapy and Follow up*), patients are being treated with TSH-suppressive therapy to inhibit TSH-dependent growth of residual cancer cells (37). Unless recurrence or metastases develop patients are then switched to euthyroidal doses of levothyroxine (20). Although long-term TSH suppression may be associated with an overall better prognosis in high-risk patients (37), harmful effects on the cardiovascular system, carbohydrate metabolism, psychological well-being and bone mineral density may be present (37). Studies about the adverse effects on bone mineral density have been inconsistent, possibly because of the different lengths of treatment, administered dosages, methodologies or sample sizes. Most studies did not find adverse effects in premenopausal women or men (150-155), whereas the effects on postmenopausal women are inconsistent (150-152, 156).

Cardiovascular system

A recent published retrospective cohort study showed that the risk of cardiovascular mortality is increased 3.3-fold in patients with DTC compared with controls, independent of age, sex, and cardiovascular risk factors (157). Each 10-fold decrease in the geometric mean TSH level is independently associated with a 3.1-fold increased risk of cardiovascular mortality. Another recent study in mortality in DTC showed that cardiac and cerebrovascular diseases are the most frequent causes of non-cancer mortality (158). Long- term subclinical hyperthyroidism in middle aged individuals results in increased heart rate, increased left ventricular mass (LVM), increased mean arterial pressure, and diastolic dysfunction while in elderly patients (>60 years old) supraventricular arrhythmias, including atrial fibrillation are prevalent (37, 159, 160).

Increased LVM with left ventricular concentric remodelling in long-standing subclinical hyperthyroidism leads to systolic dysfunction (161, 162), and is associated with increased mortality (163).

In **Chapter 7**, we analysed the effects of prolonged TSH suppressive levothyroxine therapy on cardiac dimensions and function using 2-dimensional (2D) speckle tracking echocardiography, which permits the study of multidirectional active deformation of the myocardium (radial, circumferential and longitudinal strain and strain rate), providing comprehensive information on myocardial function (contractility) (164-166).

During levothyroxine withdrawal, patients are repeatedly exposed to short-term hypothyroidism. Young- and middle-aged patients with short-term hypothyroidism show reduced exercise capacity and an increased prevalence of hypertension. In patients with pre-existing cardiovascular diseases, their condition worsens (78). Short-term hypothyroidism mostly results in impaired diastolic and/or systolic function (78, 167-174). Most of these cardiovascular alterations are reversible after reinduction of levothyroxine therapy (78, 175).

In **Chapter 8**, using 2D speckle tracking echocardiography, we analysed changes in left ventricular function in DTC patients in transition from exogenous subclinical hyperthyroidism via euthyroidism to overt hypothyroidism.

Health-related quality of life (HRQOL)

It is obvious that a diagnosis of cancer has a major impact on psychological well-being. This is evident in patients with active and progressive disease. As DTC is considered a malignancy with an overall limited aggressiveness, until recently, it was not suspected that cured DTC patients may suffer from impaired quality of life, even as these patients were sometimes subjected to lifelong follow-up.

In a systematic review it was found that thyroid cancer survivors generally have a similar or worse HRQoL compared with controls (176).

In a subsequent large controlled study, the same authors found that long-term thyroid cancer survivors experienced more symptoms and deteriorated HRQoL compared to the normative population (177). Interestingly, it was found that thyroid cancer survivors experienced several areas of information provision as insufficient, which could contribute to their diminished quality of life suggesting room for improvement (178).

OUTLINE OF THE THESIS

In this thesis several basic and clinical questions regarding diagnosis, therapy and adverse effects of therapy in DTC are addressed in clinical and preclinical studies.

In **Chapter 2**, we describe an immmunohistochemical study in patients with benign and malignant thyroid disorders to explore the diagnostic value of BMP-7 and its signalling pathway.

In Chapter 3 we study the hypothesis of a short negative feedback effect of thyroid hormone on the regulation and expression of the sodium iodide symporter (NIS).

In **Chapter 4** we describe the long-term efficacy and safety of the multikinase inhibitor sorafenib in patients with advanced DTC that are no longer RAI responsive.

In **Chapter 5**, we study the hypothesis that sorafenib induced hypothyroidism is related to alterations in enhanced metabolism of levothyroxine through induction of type 3 deiodinase.

In **Chapter 6** we describe mechanistic studies aimed at the improvement of functional NIS expression by interfering with AMPK

In **Chapters 7 and 8,** the consequences of TSH suppressive levothyroxine dosages as well as short term hypothyroidism on the cardiovascular system are being described, using dedicated cardiac ultrasound.

Finally, **Chapter 9** provides a summary of the results and a discussion of their implications, followed by a Dutch translation in **Chapter 10.**

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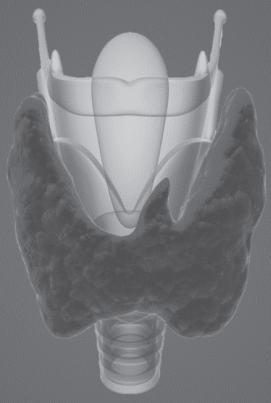
CHAPTER 2

Expression of the Bone Morphogenetic Protein 7 Pathway in Differentiated Thyroid Disease, An Immunohistochemical Study



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ABSTRACT

Objective: bone morphogenetic proteins (BMPs) are important regulators of growth and apoptosis in numerous tissues. Until now, no systematic studies on tissue expression of BMP7 and its downstream proteins (Smad4 and pSmad1) in normal and pathological human thyroid tissues have been performed. We conducted an immunohistochemical study aiming for exploring the differential expression of these 3 proteins in normal and diseased thyroid tissues.

Methods: a tissue array was prepared containing 750 thyroid samples from 118 patients; benign thyroid tissues from 38 patients (multinodular goiter, graves disease and follicular adenoma (FA)) and non-medullary differentiated thyroid carcinoma tissues (DTC) from 80 patients (papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC), follicular variant of PTC (FVPTC) and tumors with mixed areas of both PTC and FTC (PTC/FTC mix)]. Expression of the above mentioned proteins was studied and analyzed semi quantitatively by 3 observers independently. The differential expression was analyzed by a multilevel regression model with Bonferroni correction for multiple testing. Normal thyroid tissue was considered as a reference.

Results: In DTC, cytoplasmic BMP7, Smad4 and pSmad1 expression was increased with low nuclear pSmad1 expression. This pattern was present in all malignant subtypes except for PTC where the cytoplasmic Smad4 was over-expressed. In GD, BMP7 expression was low. In the analysis of follicular lesions, nuclear pSmad1 expression was lower in FvPTC compared with FA.

Conclusion: In conclusion, we found differentially expressed proteins of BMP7 signaling pathway in a large array of thyroid tissues. The underlying functional mechanisms remain to be established, whereas the diagnostic potential of our observations seem to be limited.

INTRODUCTION:

Bone morphogenetic proteins (BMPs) are the largest subgroup of the transforming growth factor-β (TGF-β) superfamily (1). Although the first function identified was the induction of ectopic bone formation in vivo, it has become evident that BMPs have an important function in embryonic development, and the regulation of growth, differentiation and apoptosis of various adult cell types) (2, 3). Binding of BMP7 to its receptor leads to the phosphorylation of R-Smads (Smad1/5/8), which then bind to co-Smad (Smad4) and translocate into the nucleus to regulate gene expression (3). Smad4 is the co-Smad of all the TGF-β superfamily members. Its absence or reduced expression has been found in various cancers including papillary thyroid carcinomas, which alters the TGF-β signal (4- 6). Smad4 mutations are frequent in thyroid neoplasms (7) where one point mutation (C324Y) seems to play an important role in thyroid carcinogenesis, growth and invasiveness (8, 9).

BMP7 is involved in growth and apoptosis and is expressed in many tissues (10) including the thyroid (1) and therefore, it is not surprising that aberrant expression of BMP7 has been found in a variety of diseases. The performed studies on the BMPs system in the thyroid are few (11- 16). Several BMPs including BMP7 seems to be inhibitory to thyroid function and growth (12, 13). In thyroid tumors, a missense mutation in BMP2& BMP3 overexpression seems to correlate to tumor aggressiveness (15, 16) and BMP7 has shown growth inhibitory effects in anaplastic thyroid carcinoma cell lines (11).

As neither BMP7 nor its R-Smads differential expression have been studied in thyroid; we set out to study the expression of BMP7, Smad4 and phospho-Smad1 (pSmad1), by immunohistochemistry in a large array of human thyroid tissues, including a spectrum of benign and malignant thyroid disorders, hypothesizing that a differential expression would be present, which could have potential value for the differential diagnosis of thyroid disorders.

MATERIALS AND METHODS

Thyroid tissues

We analyzed the nuclear and cytoplasmic expression of BMP7, Smad4 and pSmad1 by immunohistochemistry in 750 histological samples of 118 patients from surgically removed thyroid tissue. For all samples, the tissue representing pathological changes and their adjacent

thyroid normal tissue were obtained from the archive of the Department of Pathology of the Leiden University Medical Centre. Formalin-fixed, paraffin-embedded blocks from benign thyroid tissues of 38 patients (multinodular goiter (MNG) n=15, Graves disease (GD): n=10 , follicular adenoma (FA) n=13), and differentiated non-medullary thyroid carcinoma tissues (DTC) from 80 patients (papillary thyroid carcinoma (PTC) n=47, follicular thyroid carcinoma (FTC) n=15, and follicular variant of PTC (FVPTC) n=13 and tumors having mixed areas of both PTC and FTC (PTC/ FTC mix) n= 5) were included plus adjacent normal thyroid tissue of the pathological tissues (NT) n=69. These diagnoses were based on original histological assessment and again were reassessed by two independent pathologists before inclusion in this study. Given the variability in phenotype of follicular lesions, only microfollicular FA and widely invasive FTC's were included. Likewise, we only included encapsulated FVPTC tumors with a typical PTC nuclear pattern.

Tissue microarrays (TMA)

The selected blocks were routinely prepared from surgically removed thyroids. From each specimen 3 and up to 18 tissue cores with a diameter of 0.6 mm were taken reaching a total of 750 samples (Beecher Instruments, Silver Springs, MD, USA). They were arrayed on a recipient paraffin block, using standard procedures (19). Renal tissue samples were used as positive control for the proteins expression.

Antibodies

The following primary antibodies were used: α-human BMP7 antibody (1: 200; 2854 Ab; rabbit polyclonal directed against pro-domain of BMP7, kindly provided by Prof.Dr. S. Vukicevic (Zagreb University, Croatia); Smad4 antibody (1:200; clone B8; monoclonal Ab Santa Cruz, Santa Cruz, CA, USA) and antibody against pSmad1/5/8 (1: 100; #9511; rabbit polyclonal Antibody; Cell Signaling Technology, Danvers, MA, USA); Secondary antibodies were: biotinylated goat-anti-mouse Ig/HRP and rabbit-anti-goat Ig/HRP (1: 200; Dako Cytomation, Glostrup, Denmark). All antibodies were diluted in 1% PBS/BSA.

Immunohistochemistry

Four micrometer consecutive tissue sections were cut from each arrayed paraffin block and prepared on pathological slides. The sections were deparaffinized in xylene followed by 0.3% hydrogen peroxide in methanol at room temperature for 20 min to block endogenous

peroxidase. After rehydration, antigen retrieval was performed by microwave treatment in citrate buffer (0.01 M, pH 6.0). After 2 h of cooling, the sections were incubated overnight with the primary antibodies at room temperature. Afterwards, the sections were incubated for 30 min with the secondary antibodies. Avidin: Biotinylated enzyme Complex was applied using VECTASTAIN ABC kit (VECTOR LABORATORIES, INC. U.S.A) for 30 minutes. The antibody complexes were visualized using 0.04% diaminobenzidine-tetrahydrochloride (DAB) in 0.05-M Tris/HCl (pH 7.6) for 10 minutes in room temperature. Lastly the sections were counterstained with Mayers Haematoxylin. Negative controls were stained with the primary antibody omitted.

***Immunohistochemical* scoring**

A semi-quantitative scoring assessment of the immunostaining was performed for both the intensity and the percentage of staining for each protein both in cytoplasm and nucleus per tissue sample. Cells with negative staining were scored zero. The percentage of cells with positive staining was scored between 1 and 4 as follows: 1= >1–20%; 2= >20–50%; 3= >50–70%; and 4= >70%. The staining intensity was scored between 1 and 3 as follows: 1= faint; 2= intermediate; and 3= intense. For proportional representation of each protein expression, the percentage and intensity scores of its immunostaining were multiplied. The total score per sample therefore ranged from 0 to 12. Nuclear (n) and cytoplasmic (c) expression patterns were scored separately and registered as nBMP7, cBMP7, nSmad4, cSmad4, npSmad1 and cpSmad1. The scoring was independently conducted by three investigators (RA, HV and HH). TMA slides were viewed by the panoramic viewer 1.14.50; RTM software obtained from 3DHISTECH website (http://www.3dhistech.com/pannoramic_viewer).

Statistical analyses

The studied tissues represent a 3 levels hierarchy of: several samples per patient, patients per subcategory and lastly subcategories per main category and the differential expression of these proteins was analyzed by a multilevel linear regression analysis accounting for clustering of histological samples. Initially, quantitative score of each protein were expressed as mean ± standard error of mean (SEM) per histological category. Next, at the 1st stage for comparison of proteins expression in normal tissue versus GD, MNG, FA and malignant tissue, a total of 24 statistical tests were performed (3 proteins in 2 sites for 4 different pathological lesions). Secondly this regression model was used to compare expression in each

DTC category (PTC, FTC, FVPTC and PTC/FTC mix) vs. normal tissue, again a total of 24 tests. The 3rd stage of regression analysis compared expression in FTC and FvPTC vs. FA (12 tests). Bonferroni correction was applied to adjust for multiple testing in each stage of the analysis (B-adjustment). Differences were considered significant at an overall of $P < 0.05$ for each stage, and after B-adjustment: $p < 0.0021$ (= 0.05/24) for stage 1 and 2; $p < 0.0042$ (= 0.05/12) for stage 3. The choice for an overall p-value of 0.05 at each stage was based on the consideration that every stage represents one single hypothesis.

RESULTS

BMP-7 axis differential expression in differentiated thyroid lesions and normal tissue (Figure 1& 2):

Figure 1 shows the pattern of expression of BMP7, Smad4 and pSmad1. Semi-quantitative BMP7, Smad4 and pSmad1 expression (mean± SEM) are shown in (Figure 2).

First, in all DTC tissues combined, as well as GD, FA and MNG cytoplasmic and nuclear expression were compared with normal tissue (Figure2 A& C respectively). In GD, cBMP7 expression was lower than in normal tissue, whereas downstream proteins cSmad4 and cpSmad1 were higher than in normal tissue. With B-adjustment, only the lower cBMP7 expression in GD versus normal tissue remained statistically significant. MNG revealed only higher cSmad4 expression versus normal tissue, which was no longer significant after B-adjustment. FA had higher cBMP7 and cSmad4 expression as compared with normal tissue, which was no longer significant with B-adjustment. All malignant tissues combined revealed higher cBMP7, cSmad4, cpSmad1 expression as compared with normal tissues, whereas npSmad1 was lower.

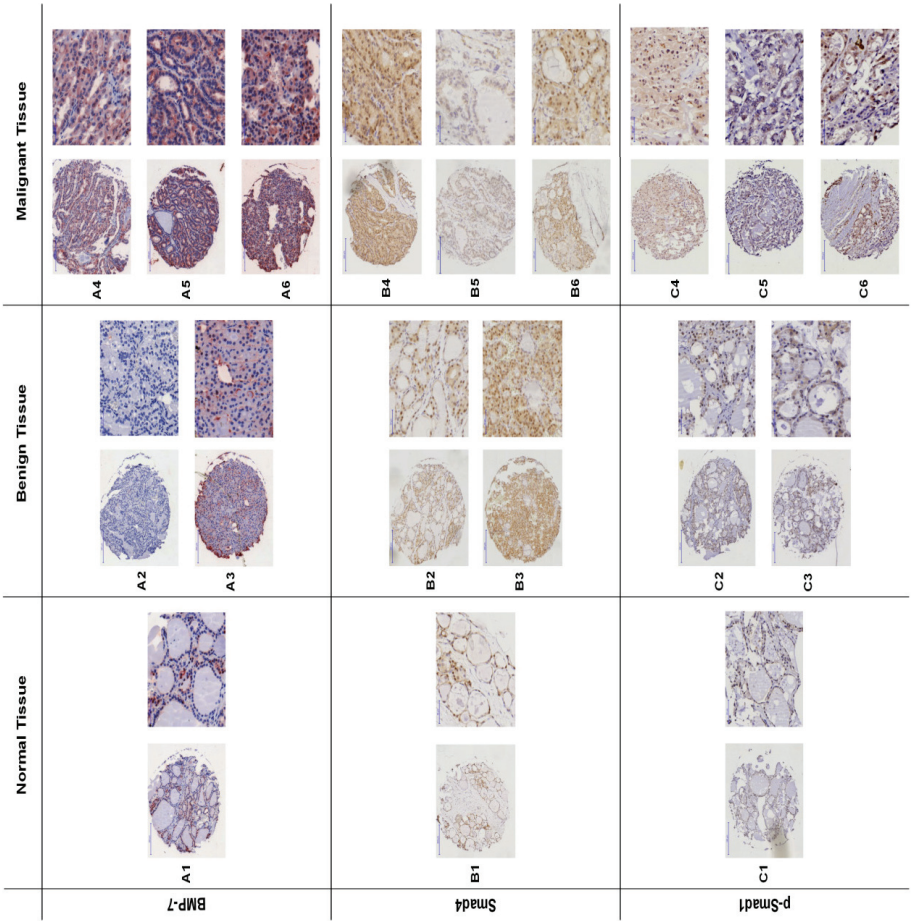

Figure 1: Immunodetection of the studied proteins is represented in 3 panels: A; immunodetection of BMP7. B; immunodetection of Smad4. C; immunodetection of pSmad1. A1, B1and C1 are sections from normal tissue (NT) (n=69). A2, B2 and C2 are sections from Graves disease tissue (GD) (n=10). A3, B3 and C3 are sections of follicular adenoma tissue (FA) (n=13), A4, B4 and C4 are sections of papillary thyroid carcinoma (PTC) (n=47). A5, B5 and C5 are sections of follicular variant of PTC tissues (FVPTC) (n=13). A6, B6 and C6 are sections from follicular thyroid carcinoma (FTC). Each tissue core is represented by two adjacent magnification powers; 10x magnification and 30x magnification. BMP-7 expression pattern is moderate patched cytoplasmic staining with few positive nuclei in NT (A1); almost absent in GD (A2); faint homogenous cytoplasmic with patches of moderate intensity and few positive nuclei In FA (A3). In malignant tissues; its expression is moderate to intense homogenous in cytoplasm of all tissues (A4-6) with 25-40% of the nuclei were positive with moderate intensity in PTC (A4) & FTC (A6) while in FVPTC (A5) less than 10% of the nuclei showed positive expression. For Smad4 immunodetection: in NT(B1), GD (B2) and FA (B3), most of the cytoplasm and about 50% of the nuclei are positive with moderate intensity. In DTC, the expression is homogeneously mild to moderate in cytoplasm and nuclei of PTC (B4) and FTC (B6) and faint to mild in the cytoplasm of FVPTC (B5) with positive expression in~40% of its nuclei. For pSmad1 immunodetection: faint patched cytoplasmic and moderate nuclear (25-40% of nuclei) expression was present in NT (C1) and FA (C3), homogenous faint cytoplasmic and nuclear (70%) expression in GD (C2). In DTC; it show homogenous moderate to faint cytoplasmic and ~50% nuclear expression in PTC (C4) and FTC (C6), while in FVPTC (C5) it is faintly scattered in cytoplasm with hardly any nuclear expression.

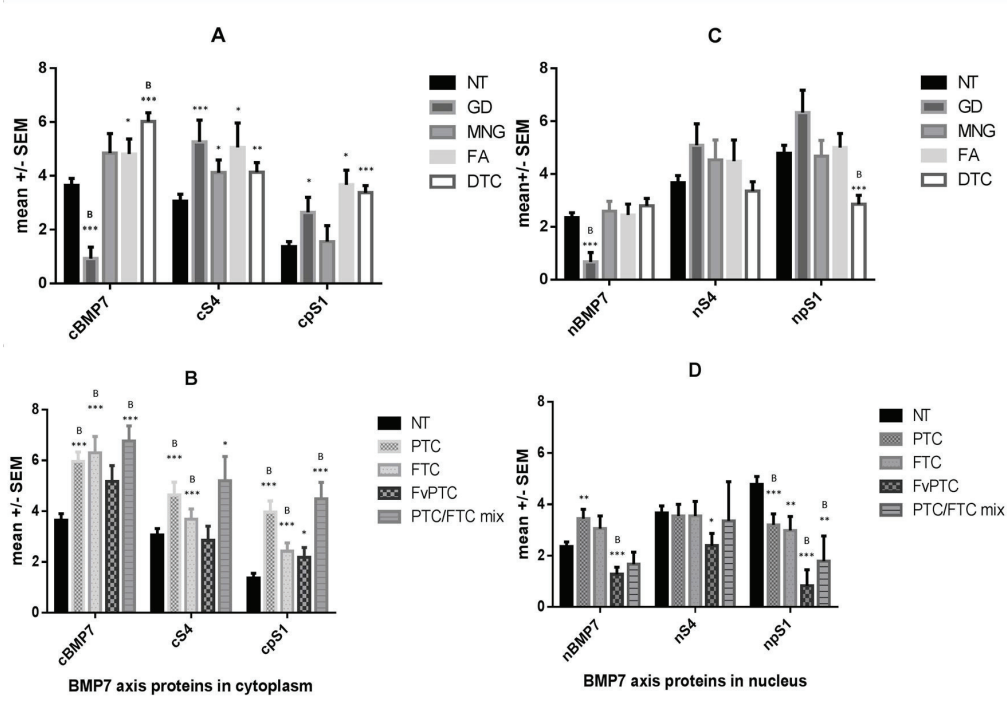

Figure 2: Expression of BMP-7 axis (BMP7, Smad4 and pSmad1) in normal (NT) and pathological thyroid tissues (mean± SEM). (A) and (C) represent Differential of cytoplasmic and nuclear expression respectively of BMP7 axis in: Graves disease (GD (n=10)), multinodular goiter (MNG (n=15)) and differentiated thyroid carcinoma tissues (DTC (n=80)) vs. NT (n=69)). (B) and (D): Differential cytoplasmic and nuclear expression (respectively) of BMP7 axis in DTC subcategories vs. NT. DTC subcategories: Papillary thyroid carcinoma (PTC (n=47)), follicular thyroid carcinoma (FTC (n=15)), follicular variant of PTC (FVPTC (n=13)) and tumors having mixed areas of both PTC and FTC (PTC/FTC mix (n=5)). *= p value<0.05, **= p value<0.01, ***= p value<0.001 and B= p value < (0.05/24) after Boneferroni adjustment.

Next, the regression model was used to compare these proteins in individual thyroid cancer categories vs. normal tissue in cytoplasm and nucleus (Figure 2 B& D respectively). With the previously detected pattern of expression seen in all malignant tissues combined vs. normal tissue (see above) as a reference in describing the expression in malignant categories: PTC showed the same differential expression pattern both with and without B-Adjustment; In FTC, cSmad4 was not differentially expressed in comparison to normal tissue and with B-adjustment also npSmad1 expression was also no longer different; PTC/FTC mix also had the same differential expression pattern as in all malignant tissues combined and with B-adjustment cBMP7 and npSmad1 remained significant. In FVPTC, BMP7 and cpSmad1 were

higher, whereas nuclear expression of BMP7, Smad4 and pSmad1 were lower than in normal tissue and with B-adjustment, only nBMP7 and npSmad1 remained significant.

BMP-7 axis differential expression in follicular thyroid lesions:

The 3rd level of regression analysis included the follicular lesions (FTC and FVPTC vs. FA). Morphologically, FTC and FVPTC can be difficult to distinguish from FA. In FTC, nBMP7 and cBMP7 were higher than in FA whereas cSmad4 was lower (Table 1).

However, with B-adjustment, these differences were no longer significant. In FVPTC, cSmad4, nSmad4 and npSmad1 expression were lower than in FA (Table 1) and with B-adjustment, differences in nSmad4 and npSmad1 expression remained significant.

Table 1: The differential expression of BMP-7 axis in follicular thyroid lesions (malignant vs. adenoma)

Follicular lesion	cBMP7		nBMP7		cSmad4		nSmad4		cpSmad1		npSmad1	
	mean± SEM	*p*	mean± SEM	*p*	mean± SEM	*P*	mean± SEM	*P*	mean± SEM	*P*	mean± SEM	*P*
FA (n=13)	4.81± 0.56		2.45± 0.41		5.06± 0.90		4.48± 0.81		3.66± 0.55		5± 0.54	
FTC (n=15)	6.3± 0.64	0.031	3.06± 0.49	0.04	3.68± 0.41	0.031	3.55± 0.57	0.09	2.42± 0.33	0.24	2.98± 0.55	0.07
FVPTC (n=13)	5.18± 0.62	0.26	1.28± 0.27	0.46	2.85± 0.56	0.005	2.39± 0.48	0.001[B]	2.18± 0.39	0.58	0.83± 0.62	0[B]

c: cytoplasmic; n: nuclear; FA: follicular adenoma; FTC: follicular thyroid carcinoma; FVPTC: follicular variant papillary thyroid carcinoma; *p:* p value .
[B]: remained significant after Bonferroni correction with p value < (0.05/12).

DISCUSSION

The present study was conducted to analyze the protein expression of BMP7 and 2 downstream proteins, Smad4 and pSmad1 in a large array of normal and pathological human thyroid tissues, which has not been documented before. We hypothesized that a differential expression would be present, which could have potential value for differential diagnosis of thyroid disorders. In general, we found cytoplasmic BMP7 overexpression in thyroid carcinoma, which is in line with observations in breast cancer, bone metastasis of prostate carcinoma, melanoma, osteosarcoma and ovarian cancers (17, 18). In breast carcinoma and

prostate carcinoma, BMP7 expression was associated with increased aggressiveness. This finding seems paradoxical, as experimental studies revealed that BMP7 is an important inhibitor of TGF-β induced epithelial mesenchymal transition, a process involved in the formation of distant metastases (19, 20). However, BMP7 protein expression may not be necessarily associated with increased activity. As in our study, nuclear expression of pSmad1, an important downstream protein in the BMP7 signaling cascade was consistently decreased in all tumor types (together with increased cytoplasmic pSmad1 expression) which may point to decreased activity of the BMP7 pathway in thyroid carcinoma. Smad4 is a key mediator downstream protein of the transforming growth factor-β (TGF-β) superfamily members (21). In line with Matsu etal, 2010 (22), we found mild/moderate cytoplasmic over expression of Smad4 in PTC and FTC. Yet the fact, Smad4 gene carry a wide range of mutations in both benign and malignant thyroid tumors that yields different phenotypes ranging from no- to over-expression (7) render the IHC staining *per se* non reflective to the mutational Smad4 status.

The association between decreased BMP7 pathway activity and progression of cancer may implicate that the BMP7 pathway may be an attractive target for future interventions in thyroid cancers. Indeed, experimental studies in several tumor types have revealed beneficial effects of BMP7 treatment (23- 26) including the anaplastic thyroid carcinoma cell lines where BMP7 treatment showed growth inhibition (11). Unfortunately, our study did not include any anaplastic or medullary thyroid cancer tissue, which might have shed light on the expressional pattern of this pathway in such aggressive cancers.

In addition to potential therapeutic implications of BMP7 expression in thyroid carcinoma, protein expression of the BMP7 pathway may also be helpful in the differential diagnosis of follicular neoplasm. Unfortunately, we found no differences in BMP7 or downstream proteins between FTC and FA with diagnostic potential. However, the decreased npSmad1 expression in FVPTC vs. increased expression in FA may be worthwhile to explore in further large and multi-center studies

Interestingly, decreased nuclear and cytoplasmic BMP7 expression was found in GD. As in GD, TSH receptors (TSHR) activation by stimulating autoantibodies is the underlying pathogenetic mechanism; the observed effects on the BMP7 pathway in our study are likely secondary phenomena. A mutual functional relationship between BMP signaling and TSH has

been suggested in experimental studies: BMP7 was shown to inhibit TSH receptor expression, whereas TSH selectively inhibits BMP signaling (12, 13). In GD, decreased, rather than increased nuclear expression of pSmad1 would have been expected, which was however not observed in our study. At this point no definite conclusions on the relationship between TSHR activation and BMP signaling in GD can be reached from our observations and further functional studies are needed to affirm this relation.

In conclusion, we found differentially expressed proteins of the BMP7 signaling pathway in a large array of normal, benign and malignant thyroid tissues. The underlying functional mechanisms remain to be established, whereas the diagnostic potential of our observations seems to be limited.

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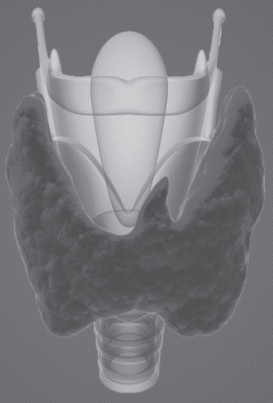
CHAPTER 3

Sodium Iodide Symporter (NIS) modulation by Tri-iodothyronine (T3) in the Thyroid



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This Thesis

ABSTRACT

Background: Thyroid hormone production by the thyroid is regulated by a negative feedback-loop (Hypothalamic-Pituitary-Thyroid (HPT) axis). Even though thyrocytes exhibit thyroid hormone transporters and receptors, the evidence for direct regulatory effects of THs on thyroid function itself remain scarce.

Aim: Our main objective was to determine if and how, T3 directly affects iodide uptake and whether this corresponds to NIS levels. Furthermore, we aimed to elucidate some of the underlying mechanisms through which T3 modulates thyroid function within thyroid follicular cells.

Methods: Fischer rat thyroid cells (FRTL5) were treated with T3 (1nM, 10nM & 1μM) in the presence of TSH for 1 to 4 days. The effects of T3 on NIS protein levels and iodide uptake were examined by western blotting and radioactive iodide uptake respectively. The effect of T3 on 5' adenosine monophosphate activated protein kinase (AMPK) activity modulation in thyroid cells was examined by western blotting. Rat NIS promoter constructs were transiently transfected into FRTL5 cells to study the possible genomic regulatory action of T3 on NIS gene expression.

Results: Radioactive iodide uptake in FRTL5 cells was significantly reduced at the highest concentration of T3 (1μM) in 6H medium (12% (24h) and 35% (48h) reduction vs. basal respectively) ($p<0.05$). After 4 days of T3 treatment the effect on iodide uptake became significant at 1nM with a 30% reduction vs. basal ($p<0.05$). NIS protein levels in FRTL5 cells were significantly decreased in a dose dependent manner after 4 days of T3 (1nM-1 μM). NIS promoter activity was significantly reduced in a dose dependent manner. T3 decreased NIS promoter activity to 67.8%±12 (1μM); $p<0.01$ in comparison to control at day1. At day 4, the reduction in promoter activity was 68.3%± 6.9 (1nM); $p<0.001$ and 50.1%±8.4 (1μM); $p<0.001$. No reduction in promoter activity could be detected in constructs harboring isolated regions of the NIS promoter. Furthermore, AMPK phosphorylation (p-AMPK) levels in FRTL-5 cells were not affected by T3 treatment.

Conclusion: T3 down-regulates NIS protein levels and I-uptake in follicular thyroid cells. This may be explained by the persistent reduction in NIS promoter activity after T3 treatment. However, the mechanisms leading towards the reduction in promoter activity are unknown but seem to involve regions outside the known regulatory regions such as NUE and the proximal promoter. This finding may have important clinical consequences for rhTSH aided radioactive iodide (RAI) therapy in patients with metastases of differentiated thyroid carcinoma.

INTRODUCTION

Thyroid hormone (TH) production is regulated via the negative feedback loop between the THs serum levels and the neuroendocrine system (pituitary gland and hypothalamus) (1, 2). Although follicular thyroid cells express TH receptors (TRs), T3 cytoplasmic binding sites (3) and thyroid hormone transporter (MCT8) (4),evidence for direct influence of THs on thyroid growth and function remains limited (3, 5, 6).

T3 acts either via nuclear receptors or indirectly via the modulation of cytoplasmic signalling pathways. On the genomic level, T3 acts as a nuclear regulatory factor of transcription after dimerization to its specific receptors on the nuclear membranes (TRs), an action that requires hours to days to take effect (7- 9). The NIS Upstream Enhancer region (NUE) is the main regulatory region in the NIS promoter as it contains all the known enhancers (TTF1, Pax8, CRE and NF-κB) to stimulate transcription in a thyroid specific, cAMP-dependent manner (10, 11). Previous authors have reported that T3 reduces cAMP production in the thyroid (5, 6). Mitogen-activated protein kinase superfamily members (ERK 1/2 and p38 MAPK), PKC, PI3K, Akt and AMPK are among the direct cytoplasmic targets of T3. Via this route T3 can regulate the downstream targets in these pathways. Very recently, our group and others have shown that AMPK activation, at least partially through transcriptional regulation of NIS protein gene, is able to decrease iodide uptake and NIS protein expression both in-vivo and in-vitro (12, 13). Therefore, AMPK activation by T3 in the thyroid provides a possible hypothesis for explaining T3 actions.

There is some evidence that T3 has effects on NIS protein levels and iodide uptake which are rate limiting steps in thyroid hormone synthesis (2). The effects of T3 on NIS protein and iodide uptake were studied only once, to the best of our knowledge, where T3 was found to inhibit both parameters after 48 hours of stimulation in a FRTL5 cell line (5). Besides confirming the previous effect of T3 on NIS expression and iodide uptake; we studied the effects of various T3 concentrations representing circulating(1nM), intracellular (10nM) and intrathyroidal (1μM) levels at different time points (up to 4 days) and to study the mechanisms involved. The clinical relevance of our study is that RAI therapy for DTC patients with metastases requires high TSH levels to stimulate iodide uptake. This can be realized either by discontinuation of levothyroxine therapy or by the application of recombinant human TSH (rhTSH) during levothyroxine therapy. rhTSH preparation is based

on the assumption that the continuation of thyroid hormone therapy itself has no direct effects on RAI uptake. However, this assumption has not been proven. Therefore, a further insight into the role of T3 on iodide uptake may ultimately be beneficial for this group of patients.

MATERIALS AND METHODS

Cell culture

The FRTL-5 cell-line was purchased from Health Protection Agency Culture Collections (Salisbury, UK) (14). The cells were routinely cultured in Coon's modified Ham's F-12 medium supplemented with 10% charcoal stripped calf serum and a six-hormone (6H) preparation consisting of 5 hormones (5H) (10 µg/mL human recombinant insulin (Sigma), 10 ng/mL somatostatin (Sigma), 10 nM hydrocortisone (Sigma), 5 µg/mL transferrin (Sigma) and 10 ng/mL glycyl-L-histidyl-L-lysine acetate (Sigma)) supplemented with 1 mU/ml bovine TSH (Sigma) to make 6H medium. Cells were maintained in a 5% CO2-95% air atmosphere at 37°C with a change of medium every third day, and passed every 7 days. Prior to treatment with T3, monolayers were cultured without calf serum for 8 h.

Treatment:

FRTL-5 cells were treated with T3 at concentrations of 1nM, 10nM and 1µM in presence or absence of TSH.

Iodide uptake in FRTL-5 cells

Iodide uptake was determined as described previously (15, 16). Briefly, 24 h before the assay, cells were washed and cultured in serum-free 5H or 6H medium (14). After washing twice with Hanks balanced salt solution (HBSS), cells were incubated for 30 min with 500 µL of HBSS containing 0.1 µCi of Na125I and 20 µM unlabelled NaI, with or without 80 µM of sodium perchlorate to control for specific uptake. The radioactive medium was aspirated; cells were washed twice with 1 mL of ice-cold HBSS and accumulated iodide was extracted with 1 mL of 1xSSC+ 0.1% SDS for 30 minutes. All fluids were counted in a γ-counter. Accumulated radioactivity was expressed as percentage of control.

Western blot

FRTL-5 cells were lysed in prepared in NP buffer and subsequently diluted 1:1 in Laemmli buffer (BioRad, Hercules, CA, USA). Cell lysate was subjected to SDS-polyacrylamide gel

electrophoresis and trans¬ferred to a nitrocellulose membrane (0.45 μm) (Pierce, Rockford, IL, USA) all subsequent steps were performed in the presence of a commercial protease inhibitor cocktail in the recommended dose (Roche, Basel Switzerland). After 20 min of blocking at room temperature, the membrane was incubated (1:1000) with the primary antibody GAPDH (sc-25778) (Santa Cruz Biotechnology, Santa Cruz, Ca, USA) at 4°C O/N followed by 1 h incubation with horseradish peroxidase conjugated secondary antibody. The immune complexes were visualized using the Molecular Imager ChemiDoc XRS System (Biorad, Hercules, CA, USA). Following equalization of the amounts of protein by comparing GAPDH expression, the following primary antibodies and dilutions were used: NIS (1:1000), a kind gift of N. Carrasco, and p-AMPK (Thr172) (1:1000) (Cell Signaling Technology, Inc., Beverly, MA, USA). NIS and p-AMPK protein expression was quantified using the Molecular Imager ChemiDoc XRS System (Biorad, Hercules, CA, USA).

Luciferase promoter reporter assays

To study transcriptional regulation of NIS expression by T3 gradient concentrations, we have obtained three promoter-luciferase constructs of rat NIS (rNIS) in PGL3 (figure 1) (10). Two constructs harbored the known functional NUE region. The NUE region is essential for initiating NIS transcription and lies between -2264bp and -2495bp upstream ATG start codon of rNIS gene. It harbours TTF1, Pax8, CRE and NF-κB responsive elements (10, 11). The first promoter, rNIS promoter-construct 1 (rNIS1) is from -1 bp to -2946 bp relative to ATG of the rNIS gene. Thus, rNIS1 harbors NUE, the proximal promoter region and flanking regions while rNIS9 only contains NUE and the proximal essential 1st 500bp of the promoter. rNIS5 (from -1bp to -564) is the region required to obtain the basal activity of NIS promoter and thus was referred to in rNIS promoter activity studies.

Full activation of transcription of NIS requires at least one Pax8 binding site and an integral CRE-L sequence (17). Also, NF-κB responsive element acts in synergism with Pax8 at the NUE region to augment activation (11). To test the involvement of CRE or NFκB elements of the NUE region in T3 actions, 2 constructs; each harboring either the CRE or NFκB elements alone were used. Renilla luciferase (pRL-CMV; Promega, Madison, WI, USA) was co-transfected to correct for transfection efficiency. FRTL-5 cells were transiently transfected immediately after seeding in 5H medium with the Luciferase constructs and

Renilla luciferase. After 24 h, the medium was replaced by fresh 6H medium with or without the addition of T3. Promoter activity (luciferase/renilla) is depicted as percentage of controls.

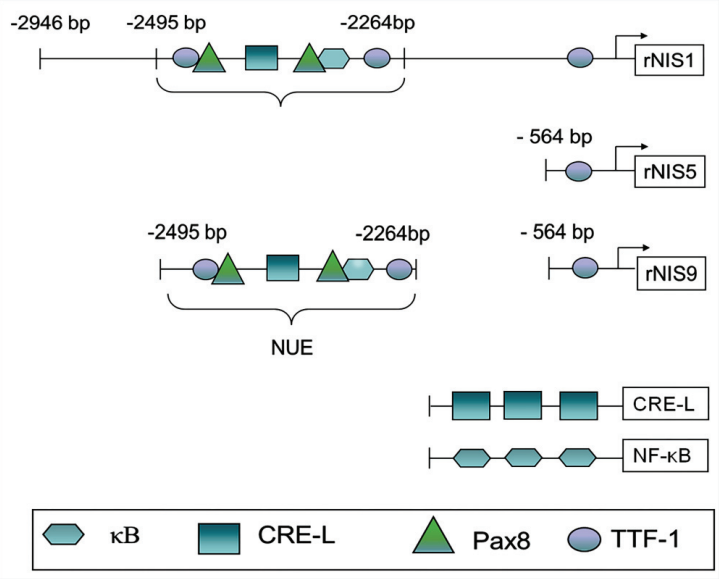

Figure1: Illustrative Overview of the constructs used in this study. Three rat NIS promoter-luciferase constructs were used; rNIS1& 9 harbour the NIS upstream enhancer (NUE) region plus flanking regions of the proximal and /or distal promoter and rNIS5 is used as a reference of rNIS promoter basal activity. NUE region contains the response elements TTF-1, CRE, Pax8 and NF-κB making it the main regulatory region for NIS expression. Two other constructs, each contains 3 repetitive sequences of either CRE-L or NF-κB responsive elements, were made to study the effect of T3 on these elements from the NUE

Statistical analyses

Results of iodide uptake and luciferase activity are expressed as proportional changes versus basal conditions. Each experiment was repeated at least 3 times. Anova tests were used for all hypotheses tested. All statistical analysis was performed using SPSS version 20.0 (SPSS, Inc., Chicago, IL). P values of <0.05 were considered significant.

RESULTS

T3 effect on iodide uptake

After 24 and 48 hours of T3 treatment iodide uptake was significantly reduced at the highest concentration of T3 (1μM) in 6H medium (12% and 35% reduction vs. basal respectively) ($p<0.05$) whereas lower concentrations did not affect the iodide uptake in FRTL5 cells. After

4 days of T3 treatment the effect on iodide uptake became significant at 1nM with a 30% reduction vs. basal ($p<0.05$) (figure 2).

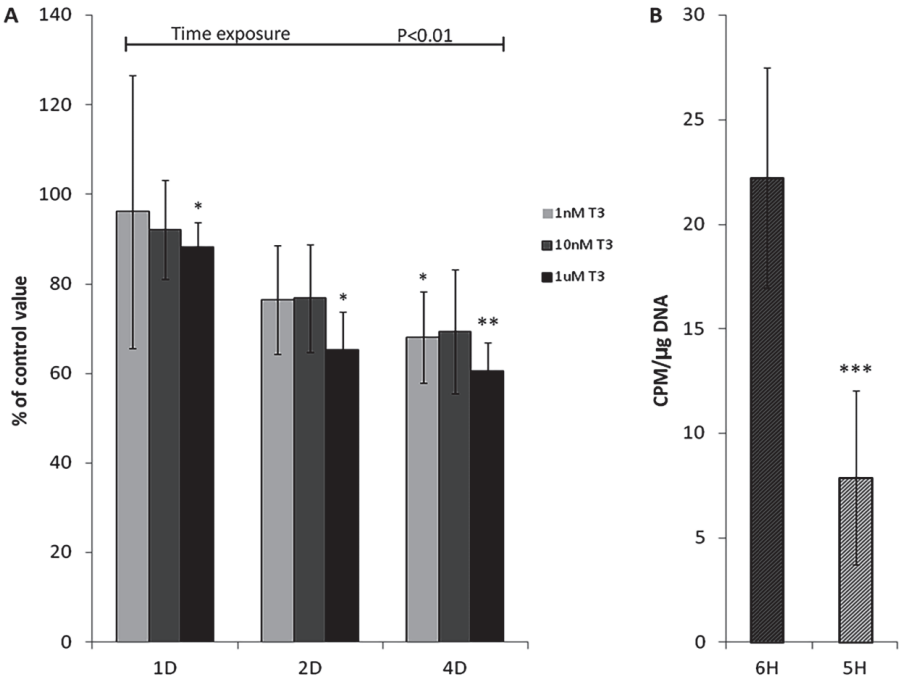

Figure2: (A) Radioactive Iodide uptake in presence of TSH in FRTL5 in response to different concentrations of T3 after 1, 2 and 4 days (1D, 2D and 4D) of stimulation. In presence of TSH, T3 significantly reduced iodide uptake through time ($p<0.01$). T3 at conc. of 1µM reduced RAI (*$p<0.05$) both at day 1& 2 in comparison to control. At day 4 this effect was detected at 1nM conc. (*= $p<0.05$) as well as the highest concentration (**=$p<0.01$) with a reduction of 60% of control value.

(B) Shows the effect of TSH on Radioactive Iodide uptake in FRTL5 cells.

Effect of T3 on NIS protein expression

To investigate whether the inhibitory effect of T3 on iodide uptake is directly related to decreased NIS protein levels, we treated FRTL5 cells with T3 (1nM, 10nM and 1µM) for 1 and 4 days in presence of TSH and analysed NIS protein expression by western blotting. T3 reduced the NIS protein expression in a time dependent manner ($p<0.001$). In addition, a clear dose dependent reduction in NIS protein levels in the cells exposed to T3 was observed at day 4 of treatment in comparison to untreated cells (figure3).

T3 and NIS promoter activity

A reduction in NIS promoter activity may be a possible explanation for the observed reduction in iodide uptake and NIS protein levels after T3 treatment. Therefore, we studied

the effect of T3 on the activity of different regions of the rNIS promoter to elucidate the effect of T3 on the known regulatory regions and to examine the possibility of other T3 responsive regions on the promoter.

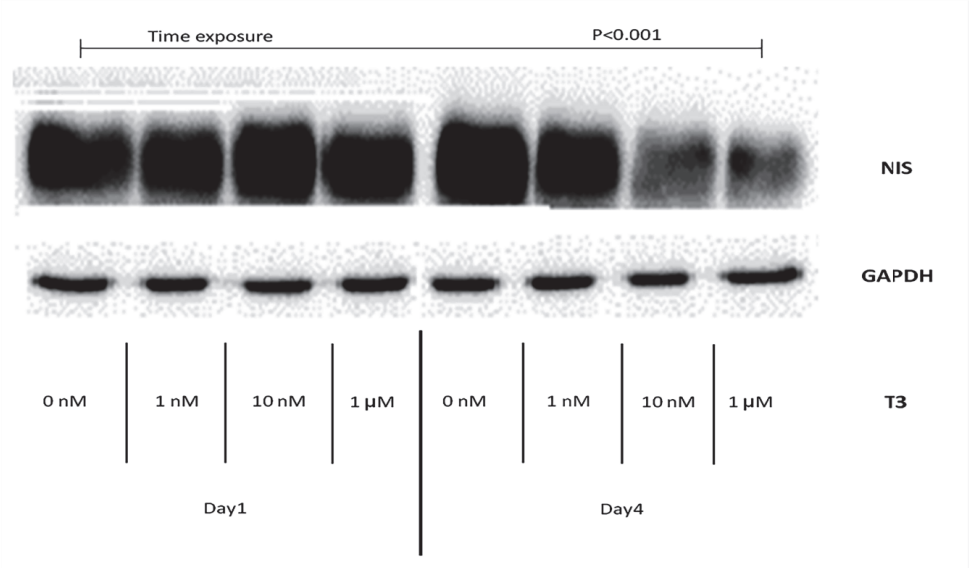

Figure3: In presence of TSH, T3 treatment resulted in reduced NIS protein expression after 4 days in a dose dependant manner. T3 reduces the NIS protein expression in a time dependent manner ($p<0.001$). The effect was evident at both the intracellular and intrathyroidal levels.

T3 exerted an inhibitory effect on the activity of the whole promoter (rNIS1) in a dose dependent manner ($p<0.001$), while the effect of time of exposure did not reach the level of significance ($p=0.053$) (figure4). The reduction (% of control value) in luciferase activity at day1 for rNIS1 at 1nM and 1µM concentrations of T3 in comparison to basal condition was 89.8% ± 27 & 67.8% ± 12; $p<0.01$ respectively. At day 4, T3 reduced rNIS1 promoter activity (to 68.3% ± 6.9; $p<0.001$ and 50.1%±8.4; $p<0.001$ at 1nM & 1µM respectively).

rNIS9 luciferase activity was not influenced by T3.

Using two luciferase promoter constructs each harboring sequences of either CRE-L or NF-κB element, we studied if T3 was able to module their activity. Neither CRE-L nor NF-κB promoter activity was affected by T3.

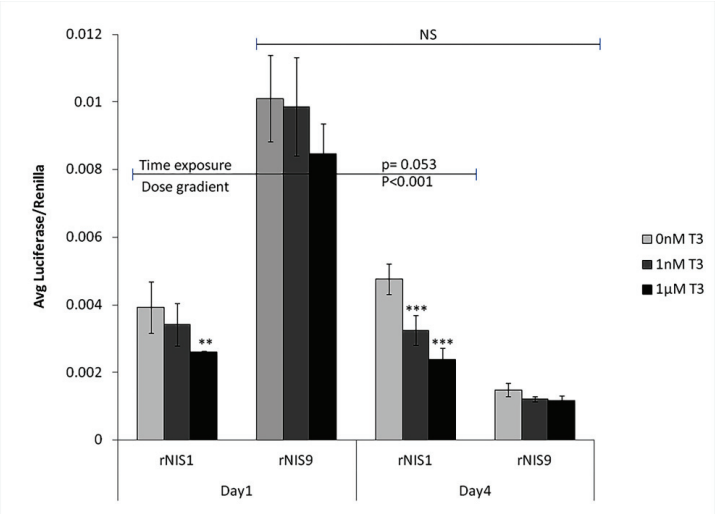

Figure4: T3 treatment of rNIS1 and rNIS9 for 1 and 4 days. Anova test analysis shows that rNIS1 luciferase activity is significantly inhibited in a dose dependent manner ($p<0.001$), an inhibition that was detected from day1 (**=$p<0.01$) in comparison to control and remained inhibited through the 4days time course treatment (***=$p<0.001$ both at 1 nM and 1µM concentrations). rNIS9 luciferase activity did not change neither in response to concentration gradient of T3 nor to the time of exposure.

T3 and AMPK phosphorylation

Because AMPK activation by T3 is a potential mechanism to explain the observed reduction in iodide uptake and NIS protein levels we have measured AMPK phosphorylation (thr^{172}) in FRTL-5 cells after T3 stimulation by western blot (12) After 4 days of stimulation, T3 in presence of TSH had no effect on p-AMPK (figure5).

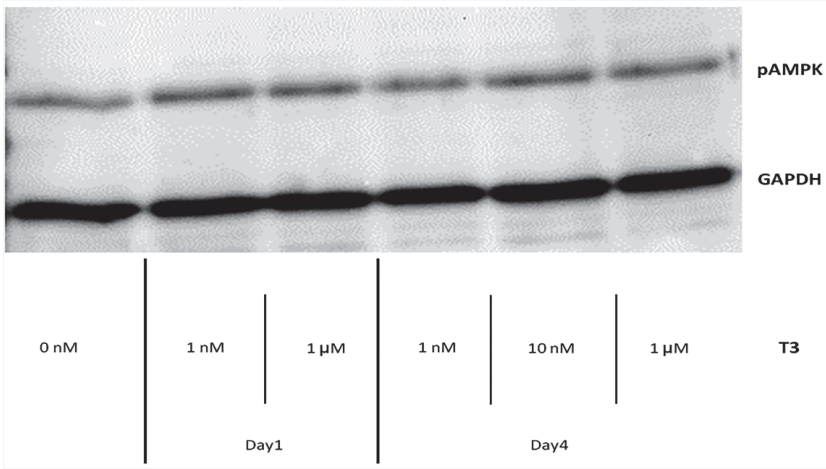

Figure5: T3 treatment in presence of TSH for 1 and 4 days did not alter the p-AMPK levels in FRTL5 cells

DISCUSSION

In the current study the effects of T3 on iodide uptake and NIS protein expression were studied.

In accordance with previous studies, T3 (1μM) was able to inhibit iodide uptake after 48 hrs (3, 6). In addition we have studied the time and concentration dependent effects of T3 on NIS protein and iodide uptake. At the highest (intrathyroidal) concentration of 1 uM, iodide uptake and NIS promoter activation significantly decreased after 1 day although no effect could be observed on NIS protein levels. As stated, this T3 concentration reflects the in vivo situation within the thyroid, which suggests that intrathyroidal T3 effects on iodide uptake may occur within 1 day. After 4 days the effects on iodide uptake and NIS promoter activity were amplified and could also be observed at 1nM T3 (circulating levels). These effects were accompanied by decreased NIS protein levels.

The partially delayed effects on NIS protein levels may be explained by the relatively long half-life of NIS protein (~ 3- 5 days in absence or presence of TSH respectively) (18).

The possibility of a genomic effect of T3 on the NIS promoter was investigated via transient transfection of FRTL5 cells with promoter reporter assays. In our experiments, the whole promoter region (rNIS1) is regulated in a dose and time dependent manner by T3. However, we did not find any T3 effects in promoter constructs which contain isolated elements of the NIS promoter region (CRE and NF- κB) nor in rNIS9 that contains only the known regulatory regions. These data point towards the involvement of regions outside the known regulatory regions. A possibility would be the presence of T3 regulatory responsive regions within the NIS promoter but outside the NUE region.

T3 actions also involve non-genomic actions via different cytoplasmic pathways (19). Increased AMPK phosphorylation at Thr172 and the subsequent activation of its signalling pathways is one of these actions. Recently our group has shown that the AMPK signalling pathway is also involved in NIS protein and iodide uptake regulation in thyroid follicular cells both *in-vivo* and *in-vitro* (12, 13).

Our data show that this non-genomic pathway is not involved in thyroidal T3 actions because in the presence of TSH, T3 did not have any effect on p-AMPK levels in FRTL5 cells.

In short, T3 inhibitory action on iodide uptake and NIS protein is dependent on the T3 concentration and requires days to manifest itself. Our results may explain the observed down regulation of NIS and I-uptake by the persistent reduction in NIS promoter activity after T3 treatment. This reduction, in addition with the relatively long half-life of NIS protein, will lead to a reduction in NIS protein over several days and subsequently in reduced I-uptake. However, the mechanisms leading towards the reduction in promoter activity are unknown but seem to involve NIS promoter regions outside the known regulatory regions such as NUE and the proximal promoter.

Our data will need to be verified in *in vivo* and human studies before we can conclude whether, or to what extent, T3 affects NIS expression and iodide uptake *in vivo*. If so, the impact of rhTSH therapy on RAI may need to be revised as in present practise it is assumed that T3 in itself does not influence iodide uptake.

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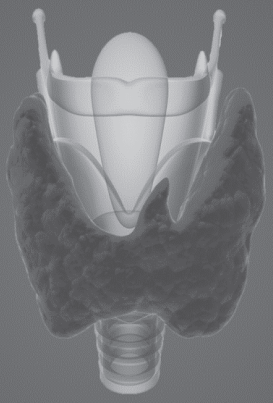
CHAPTER 4

Long-term analysis of the efficacy and tolerability of sorafenib in advanced radio-iodine refractory differentiated thyroid carcinoma: Final results of a phase II trial



Schneider TC
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Eur J Endocrinol. 2012;167(5):643-50

ABSTRACT

Objective: We conducted a prospective phase II clinical trial to determine the efficacy of sorafenib in patients with advanced radio-iodine refractory differentiated thyroid cancer. In this article, the long-term results are presented.

Patients and methods: Thirty-one patients with progressive metastatic or locally advanced radioactive iodine refractory differentiated thyroid cancer received sorafenib 400 mg orally twice daily. The study end points included response rate, progression-free survival (PFS), overall survival (OS), and best response by Response Evaluation Criteria in Solid Tumors criteria 1.0, and toxicity

Results: Median PFS was 18 months (95% confidence interval (95% CI): 7–29 months) and median OS was 34.5 months (95% CI: 19–50 months). Eight patients (31%) achieved a partial response and 11 patients (42%) showed stable disease after a median follow-up of 25 months (range 3.5–39 months). Toxicity mostly included hand foot syndrome, weight loss, diarrhea, and rash.

Conclusion: Sorafenib has clinically relevant antitumor activity in patients with progressive metastatic or locally advanced radio-iodine refractory differentiated thyroid cancer. Sorafenib can nowadays be considered as the standard option in these patients.

INTRODUCTION

Thyroid cancer is the most prevalent endocrine malignancy, accounting for 95% of cancers of the endocrine system and 66% of endocrine cancer mortality in 2010 (1). The current incidence in Europe is 49/1 000 000 per year, with a nearly three times higher incidence in women (2).

Differentiated thyroid carcinoma (DTC) is by far the most common (95%) subtype and includes papillary thyroid carcinoma (PTC, 80%) and follicular thyroid carcinoma (FTC, 10–15%) as well as subtypes like Hurthle cell carcinomas. The majority of DTCs are slowly progressive, and, when identified at an early stage, frequently cured with adequate surgical management and radioactive iodine 131-I (RAI) ablation therapy. However, metastatic DTC that has become inoperable or refractory to RAI therapy is associated with a less favorable prognosis, as 10-year survival varies between 25 and 40% (3, 4). The efficacy of conventional chemotherapy in DTC is negligible, and chemotherapy is therefore no longer recommended in international guidelines (5, 6). As a result of increased understanding of thyroid tumorigenesis, potential targets, and novel therapeutic agents that target biological abnormalities have been identified.

In DTC, the role of activated genetic aberrations in the RET–RAS–RAF–MAPK signalling pathway in tumor development and progression has been determined. The B-type Raf kinase (BRAF) V600E mutation has been reported in 29–69% of PTC and is associated with recurrent and persistent disease with a higher rate of lymph node metastasis and higher TNM stage (7, 8). In a review of 11 studies in thyroid cancer, up to 50% of follicular and 12% of Hurthle cell malignancies harbored RAS mutations or RAS downstream signalling PIK3CA mutations (9). Rearrangements in the RET proto-oncogene occur in up to 25% of PTC, resulting in the generation of chimeric oncogenes responsible for the initiation of tumor formation (10). Additionally, RAS mutations and RET/PTC translocations also result in aberrant signalling through BRAF. Furthermore, the RET–RAS–RAF pathway leads to vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR) synthesis through interconnection with the epithelial growth factor receptor (EGFR)-activated cascade (11). In turn, these are the most important regulators in the process of angiogenesis. Increased vascularity plays a crucial role in tumor growth and generating metastasis (12). In human thyroid cancer, VEGF overexpression is associated with increased tumor stage and invasiveness (11). Pazopanib is a tyrosine kinase inhibitor (TKI) that targets VEGFR1–3 and

c-KIT. Its antitumor activity in advanced and progressive DTC was demonstrated in a phase II trial, which showed a partial response (PR) rate in 49% of patients (n=39). Median progression-free survival (PFS) was 12 months (13, 14). XL184 (cabozantinib) is an oral inhibitor of RET, c-MET, and VEGFR1 and 2. c-MET activation triggers tumor growth and angiogenesis. Moreover, in patients with PTC and MTC, overexpression and frequent mutations of the c-MET receptor have been reported (15). Antitumor activity of cabozantinib in patients with DTC was recently reported at the 2012 ASCO Annual Meeting. Cabanillas et al. found confirmed PR and stable disease (SD) in 53 and 40% of patients respectively (n=15). Disease control rate (PR+SD) was 80% at 16 weeks. At the time of data presentation, median PFS and overall survival (OS) had not been reached (16).

E7080 (lenvatinib) is also an inhibitor of multiple TK, especially VEGFRs, c-KIT, and platelet-derived growth factor receptor b stem cell factor receptor. In animal studies, it has been shown to have potent antitumor activity against small-cell lung cancer and breast cancer most likely through inhibition of VEGFR2 and 3 (17, 18). A phase II trial of lenvatinib in RAI refractory DTC (n=58) by Sherman et al. (19) showed a PR rate of 50% and a median PFS of 13 months. A recent study by Rajoria et al. (20) showed estrogen to be a key regulating factor of VEGF expression in thyroid cells, suggesting a role for antiestrogens in the therapeutic regimen to treat thyroid cancer.

As a result of this increased understanding of the biological basis for thyroid cancer development, multiple clinical trials with multi-target TKIs have been conducted. Sorafenib (BAY 43-9006) is an orally active TKI that targets BRAF, VEGFR1 and 2, and RET, implementing antiangiogenic and proapoptotic actions. Results of an update of a phase II study on sorafenib, reported at the 2011 ASCO Annual Meeting by Brose et al., showed a PFS of 24 months and an OS of 35 months in 47 patients with DTC and poorly differentiated thyroid cancer (PD; n=55). A PR was achieved in 38% of patients and 47% had SD (21). Another phase II study in mainly patients with advanced DTC (n=30) reported a PR rate of 23%, an SD rate of 53%, and a median PFS of 20 months (22). Kloos et al. examined the effect of sorafenib mainly in patients with metastatic PTC (n=41) and reported a PR and SD of 15 and 56% respectively. The median PFS was 15 months (23). Recent results of a study with the MEK1/2 inhibitor selumetinib showed reinduction of radio-iodide uptake in 60% of patients with RAI refractory thyroid cancers of follicular origin (24).

Our phase II study was designed to assess the effects of sorafenib on restoring the susceptibility to RAI therapy, tumor progression, and safety in RAI refractory DTC. The first report of this study by Hoftijzer et al. (25) showed a promising efficacy and tolerability of sorafenib, but no beneficial effects on radioactive-iodide uptake. Here, we report the long-term outcomes of this sorafenib trial in advanced RAI refractory DTC with results on efficacy, safety, and tolerability.

PATIENTS AND METHODS

Patients

Details of study design and patient eligibility of this study have been described previously (25). Briefly, eligibility criteria were the presence of progressive metastases or unresectable local recurrence of DTC for which RAI therapy was no longer effective, as indicated by prior negative post-therapeutic whole-body scintigraphy. Progressive disease was defined according to Response Evaluation Criteria in Solid Tumors (RECIST) criteria 1.0 12 months before initiation of treatment. Patients had undergone total thyroidectomy and RAI ablative therapy. Eastern Cooperative Oncology Group performance status had to be <2 and life expectancy had to be more than 3 months. Written informed consent was provided by all patients before enrolment onto the trial. The study protocol was approved by the Institutional Review Board of the Leiden University Medical Center. This study was registered at Clinical-Trials.gov (#NCT00887107).

Study design

We performed a nonrandomized, open-arm, phase II trial of sorafenib in 31 patients with advanced RAI refractory DTC. For a complete description of study design, we refer to the paper of Hoftijzer et al. (25). The primary objective of this study was to determine the efficacy of sorafenib treatment with a PR as the primary end point. The secondary objective was to study RAI reuptake and the safety of sorafenib as an antitumor drug for advanced RAI refractory DTC patients. Sorafenib was administered at a dose of 400 mg orally twice a day until disease progression, uncontrollable side-effects, death, or patients' own request. Safety assessments consisted of physical examination, documentation of adverse events (AEs), and laboratory parameters (total blood count, electrolytes, and kidney and liver function) and were

performed every 4 weeks. The incidence, grade, and relationship of AEs to the drug were graded with the use of Common Terminology Criteria for Adverse Events (CTCAE, version 3.0).

After completion of 6-month treatment, patients were allowed to continue sorafenib treatment when a favorable response (complete remission, partial remission, or SD) had been achieved. Sorafenib was thereafter continued until progression according to RECIST 1.0 criteria with computed tomography (CT) scanning performed every 6 months.

In this article, we report the long-term outcomes and tolerability of sorafenib in patients with advanced RAI refractory DTC.

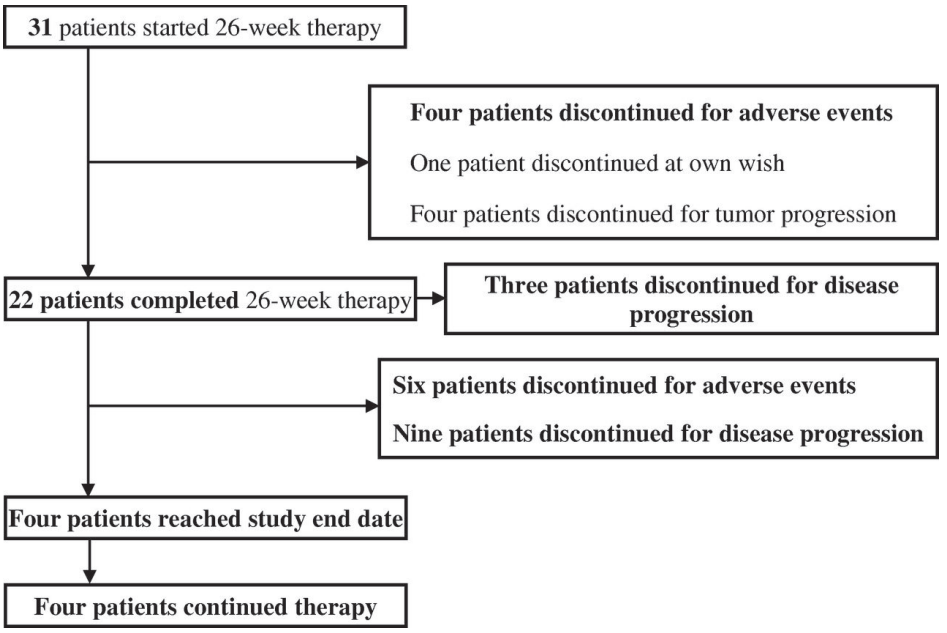
Statistical methods

In this study, data are reported as median± S.D., median (range), or proportions. Best objective response rate (RR) refers to the proportion of patients who had the best response rating for complete response (CR) or PR according to RECIST 1.0. The OS, PFS (defined as the time from starting study drug to progression), and overall disease control rate with associated 95% confidence intervals (95% CIs) were obtained using the Kaplan–Meier method. AEs were scored according to the National Cancer Institute's CTCAE, version 3.0. Other safety parameters, including laboratory data, were summarized descriptively. Variables possibly influencing response to sorafenib were analyzed with binomial logistic regression. The calculations were performed using SPSS 17.0 for Windows (SPSS, Chicago, IL, USA).

RESULTS

Patient characteristics

All 31 patients were included in this long-term outcome study on sorafenib. The follow-up of the study ended on 1 March 2011 with five patients excluded from the efficacy analysis because they did not reach the first radiological evaluation at 6 months. One patient requested to stop sorafenib treatment after 4 days, one patient developed small-cell lung cancer, and three patients discontinued treatment because of AEs. Median follow-up period was 25 months (range 3.5–39 months) and median sorafenib treatment period was 9.2 months (range 0.1–39 months). By the time the follow-up of the study ended (March 2011), four patients (15.4%) were still on sorafenib (Fig. 1).



Baseline characteristics of the included patients are listed in Table 1. As can be expected, Hurthle cell metaplasia, which is associated with poor RAI responsiveness, was overrepresented in the patient group (42%). Two of 13 PTC patients had a poorly differentiated carcinoma. A total of 15 mutations were identified on primary tumor material, of which BRAF V600E was most frequently observed with 10/13 PTC patients harboring a BRAF V600E mutation.

Efficacy

Of the 31 patients enrolled in this study, 26 patients were eligible for efficacy analysis. Five patients were excluded for the reasons previously mentioned. Data on efficacy are given in Table 2 and Fig. 2.

At a median follow-up of 25 months, the overall disease control rate was 27%. The total proportion of PR was 31% (n=8), which were all achieved in the first 6 months of treatment. Four patients (15%) had an ongoing PR and three patients (12%) showed ongoing SD. There was no CRs. Individual data per patient response is summarized in Table 3. At the time of this data analysis, the cumulative number of patients with progressive disease was 15 (58%). Disease progression was not influenced by age, gender, or histology, including the presence of Hurthle cell metaplasia. Furthermore, the presence of a BRAF V600E mutation was not

related to disease progression. The prevalence of other mutations was too low to allow statistical analysis.

Table 1 Baseline Characteristics

	All patients (n=31)
Gender (No.; %)	
Female	12 (39)
Male	19 (61)
Age (year) (median, range)	64 (53-82)
Time from diagnosis (year) (median, range)	3.9 (0.3-18)
Histology (No.; %)	
Papillary	13 (42)
Tall cell	1 (3)
Hurthle cell metaplasia	2 (6)
Follicular variant papillary	2 (6)
Follicular	15 (48)
Hurthle cell metaplasia	11 (35)
Mixed papillary follicular	1 (3)
Initial TNM stage (No.; %)	
IB (T2N0M0)	2 (6)
IIB (T2-3 N0-1 M0)	16 (52)
IIIA (T1-3 N1-2 M0)	5 (16)
IV (any T any N M1)	6 (19)
Unknown	1 (3)
Tumor extent at study entry (No.; %)	
Thyroid bed only	1 (3)
Lungs only	8 (26)
Lungs and bone only	7 (23)
Locally advanced* and distant metastases	9 (29)
Other	6 (19)

* Including both thyroid bed (n=5) and neck lymph nodes (n=4)

Tabel 2 Efficacy Analysis

	Number of patients (% of total)
Total patients	31
Assessable patients	26
Median duration of treatment (months; range)	15 (0.1-39)
Median duration of follow up (months; range)	25 (3.5-39)
Best response by RECIST 1.0	
Complete response	0 (0)
Partial response	8 (31)†
Stable disease	3 (12)
Progressive disease	15 (58)
Overall disease control	7 (27)
Median duration of SD (months; range)	26.5 (3-38)
Median overall response duration (months; range)	29.6 (3-33)
Median PFS (95% CI)	18 (7-29)
With BM	12 (#)
Without BM	20 (13-27)
Median OS (95% CI)	34.5 (19-50)
With BM	23 (20-26)
Without BM	NR

BM: bone metastases; NR: not reached; # not available, † 4 with ongoing PR

Disease progression in soft tissue metastases or bone metastases did not significantly differ. However, the favorable responses seen in three patients with bone metastases were

based on regression of metastases other than bone, predominantly lung, whereas the bone metastases were stable. The overall median OS was 34.5 months (95% CI: 19-50 months). In the presence of bone metastases, median OS was significantly worse with 23 months (95% CI: 20–26 months) compared with the median OS of the whole group. However, at the time of this data analysis, median OS was not reached for patients without bone metastases (Fig. 2A). Age and gender did not influence OS. Estimation of median PFS was 18 months (95% CI: 7–29 months). Similarly, the median PFS was influenced by the presence of bone metastases. Median PFS was 20 and 12 months in the absence and presence of bone metastases respectively (Fig. 2B). A relation between OS and PFS and site of bone metastases could not be established as 12 of 13 patients with bone metastases had metastases localized in the axial skeleton. Hurthle cell metaplasia and reduction of drug dose did not influence PFS.

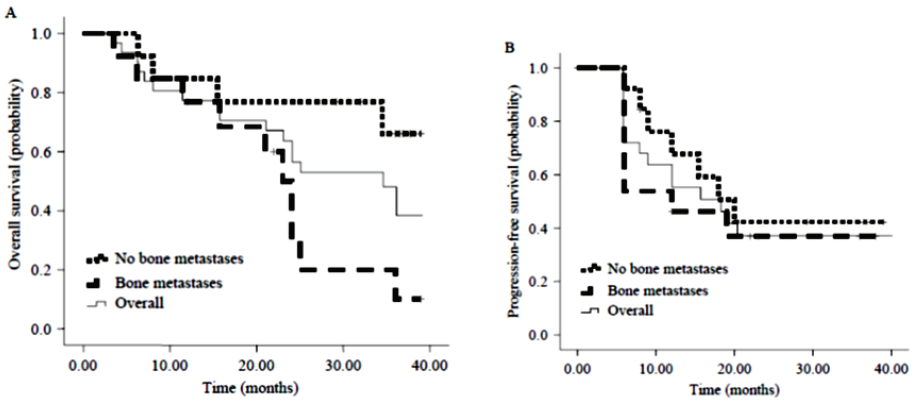


Figure 2 Kaplan–Meier curves of (A) overall survival (OS) and (B) median progression-free survival (PFS) (A) Kaplan–Meier estimate of OS. Overall median OS was 34.5 months. In the presence of bone metastases, median OS was 23 months. At the time of this data analysis, median OS was not reached for patients without bone metastases. (B) Kaplan–Meier estimate of PFS. Estimation of median PFS was 18 months. Median PFS was 20 months in the absence of and 12 months in the presence of bone metastases.

Thyroglobulin (Tg) response reflected the radiological response (Table 4). From baseline, patients with a PR had a median decrease of 24 mg/l (range, - 2746 to 2.6 mg/l) in their serum Tg levels. Patients with SD showed a median elevation of 19 mg/l (range, - 60 to 3724 mg/l). The serum Tg levels in patients that showed PD increased by a median of 49 mg/l (range, - 5320 to 87 736 mg/l). The 54-year-old woman with pulmonary metastases of a Hurthle cell FTC reported at 6 months with high Tg levels (99 900 mg/l) but a decrease in number, size,

and density of all metastases died 2 months later from disease progression with a Tg level that remained high (89 400 mg/l).

Table3 Data on clinical benefit per patient

Response	Histology	Initial tumor stadium	Duration of response (months)
PR	Papillary	IB	33[a]
PR	Follicular with Hurthle cellmetaphasia	IIB	33[a]
PR	Papillary	IIB	33[a]
PR	Papillary	IV	30
PR	Follicular	IIB	24
PR	Follicular	IIB	24
PR	Papillary	IIB	18[a]
PR	Papillary	IIIA	6
SD	Papillary	IIIA	38
SD	Follicular	IIB	36
SD	Follicular with Hurthle cell metaphasia	IIB	12

PR: partial response; SD: stable disease; IB (T2N0M0); IIB (T2-3 N0-1 M0); IIIA (T1-3 N1-2 M0); IV (any T any N M1).
[a]Ongoing response

Table 4 Thyroglobulin response per patient response

RECIST 1.0	CR	PR	SD	PD
Baseline serum Tg levels (median, range)	Non	50 (4.5 to 3126)	41 (2 to 1817)	382 (26.5 to 8570)
Last serum Tg levels (median, range)	Non	19 (0.9 to 1894)	94 (14 to 4140)	373 (38 to 89 400)
Delta Tg (vs baseline; median, range)	Non	-24 (-2746 to 2.6)	19 (-60.8 to 3724)	49 (-5320 to 87 736)

RECIST, Response Evaluation Ceiteria in Solid Tumors; Tg, thyroglobulin; CR, complete response; PR, partial response; PD, progressive disease.

Treatment tolerability and adverse events

Eighteen patients (58%) required dose reduction due to toxicity. Four of them (13%) discontinued the study later on due to AEs. Of these, three patients suffered from a myocardial infarction after 1, 20, and 22 months of sorafenib treatment respectively and one patient quit due to complaints of malaise after 39 months. Forty-four percent of dose reductions occurred in the first month of treatment. Further details on dose reduction and daily sorafenib dose are listed in Table 5. In total, seven patients (23%) terminated study participation as a result of drug-related AEs. There were three patients who discontinued sorafenib before dose reduction

after 1, 12, and 19 months due to angioedema, congestive heart disease, and malaise and weight loss respectively. One patient discontinued treatment after 3 months due to the development of small-cell lung cancer. One patient was diagnosed with carcinoma of the tongue after 46 months of sorafenib treatment. Thirteen patients (42%) required levothyroxine dose adjustments in order to maintain euthyroidism.

Table 5 Daily sorafenib dose and dose reduction

	3 months	6 months	12 months
Dose reduction (n, %)	13 (42)	16 (52)	18 (58)
Daily sorafenib dose (mg)			
Mean (± S.D.)	671 (± 198)	584 (±230)	562 (± 175)
Median (range)	600 (200- 800)	600 (200- 800)	600 (400- 800)

All observed AEs are listed in Table 6. Treatment related AEs were predominantly of grade 1 or 2, with the most common events including hand foot syndrome (HFS), weight loss, diarrhea, and rash. Grade 3 AEs consisted of HFS (23%), weight loss (10%), diarrhea (6%), rash (16%), mucositis (10%), and congestive heart disease (3%). Grade 4 AEs comprised myocardial infarction in three patients (10%), which led to death within 2 months after the infarction in one patient. The majority of AEs were seen in the first year of treatment and were controllable with dose reduction, medication, or supporting measures (i.e. dietary consultation and additional feeding). No correlations were found between toxicity and performance state or age.

DISCUSSION

The developments in the treatment of advanced thyroid cancer are a result of increased understanding of thyroid tumorigenesis. A high percentage of differentiated thyroid cancers contain aberrations in the RET/PTC–RAS–RAF–MAPK pathway. In addition, the RET–RAS–RAF pathway leads to VEGF and VEGFR synthesis through the EGFR-activated cascade. Hence, compounds targeting the activated RET–RAS–RAF pathway and beyond, antiangiogenic compounds or a combination of both, may be effective in RAI non-avid DTC (26). Several clinical trials in thyroid cancer have shown considerable percentages of clinical responses, suggesting this assumption to be accurate. Of the targeted therapies that have shown promising results in advanced differentiated thyroid cancer, only sorafenib targets both

BRAF and RET, as well as VEGFR. However, sorafenib is a relatively weak BRAF inhibitor. The most potent BRAF inhibitor, vemurafenib, might be more effective in PTC harboring a BRAF mutation and is currently under study in an international phase II trials (www.clinicaltrials.gov, NCT01286753) (27).

Table 6 Adverse events. According to Common Terminology Criteria for Adverse Events (version 3).

	All	**Grades** No. of patients (% of category)			
Event	**No. of patients (% of total (n=31))**	**1**	**2**	**3**	**4**
Hand foot syndrome	22 (71)	8 (36)	7 (32)	7 (32)	
Weight loss	18 (58)	7 (39)	10 (56)	3 (17)	
Diarrhoea	16 (52)	5 (31)	9 (56)	2 (13)	
Rash	17 (55)	11 (65)	1 (6)	5 (29)	
Alopecia	16 (52)	14 (88)	2 (12)		
Mucositis	15 (48)	11 (73)	1 (7)	3 (20)	
Hypocalciemia	15 (48)	14 (93)	1 (7)		
Hypertension	13 (42)	4 (31)	4 (31)	5 (38)	
Hypophosphatemia	11 (35)	11 (100)			
Anemia	11 (35)	11 (100)			
Hypoparathyroidism	10 (32)		10 (100)		
Thrombopenia	9 (29)	9 (100)			
Hypothyroidism	8 (26)		8 (100)		
Leukopenia	7 (23)	7 (100)			
Nausea	3 (10)	3 (100)			
Myocardial infarction	3 (10)				3 (100)
Congestive heart disease	1 (3)			1 (100)	
Hematuria	1 (3)		1 (100)		
Deep venous thrombose	1 (3)			1 (100)	
Hyponatriemia	1 (3)	1 (100)			
Pneumothorax	1 (3)		1 (100)		
Small cell lung cancer	1 (3)				1 (100)

Our study is the first study reporting the long-term effects of sorafenib treatment in advanced RAI refractory DTC patients. We also provided results of the influence of bone metastases on survival outcome and PFS. Our results were not influenced by subtypes of thyroid cancer, as we only included patients with DTC, ensuring a homogeneous study population. In our trial, we observed beneficial effects of sorafenib. At 6 and 25 months, median PFS was 14.5 and 18 months respectively. At 6 and 25 months, median OS was 25 months and 34.5 months respectively. The PR rate of 25% we observed in the first 6 months of the trial is one of the highest PR rates reported in sorafenib trials in DTC. Kloos et al. (23) found a PR rate of 15% in patients with metastatic PTC. Gupta-Abramson et al. (22) reported a PR of 23% (DTC, MTC, and anaplastic thyroid carcinoma (ATC)) whereas Ahmed et al. (28) observed a PR in 21% of patients with metastatic thyroid cancer (DTC and MTC). Our high PR rate could be attributed to the differences in patient categories (histologies, tumor stages, sites of metastases, and tumor extent), study design, and analytical methods between the different phase II trials.

The explanation for the significantly less favorable outcome in patients with bone metastases remains unclear. The relationship between the genetic profile of bone metastases and responsiveness to sorafenib is hard to establish given the lower tissue levels and difficulties in obtaining tissue from bone metastases. It can be hypothesized that VEGFR-targeted therapies are less effective in bone metastases as the role of VEGFR signal transduction in tumor expansion may differ between soft tissue and bone metastases. Another explanation may be that through systemic release of cytokines or proteins from the bone microenvironment, the presence of bone metastases influences the response to sorafenib in soft tissue metastases. The dose of sorafenib used in our study was generally well tolerated, although dose reductions were required in 58% of patients. Tumor response, however, was not affected by dose reductions. Toxicities observed in our study were mostly grades 1 and 2 and similar to those reported in the other phase II trials of sorafenib (21, 22, 23, 28, 29). A long-term safety evaluation of sorafenib in advanced renal cell carcinoma showed similar results, supporting our findings (30). Dermatological AEs were the most commonly observed. A recent study of sorafenib in patients with hepatocellular carcinoma reported better response in patients with early skin reactions (31); however, we were not able to establish that correlation. Adverse effects were in the majority of cases manageable with dose reduction, medication, or

supplementary measures. However, beneficial effects on tumor response should always be weighed against the side effects and quality of life of targeted therapies (3, 4, 29).

There are some issues that have to be addressed too. RECIST criteria should be applied for judgment of progression for inclusion in trials and for follow-up scans to determine treatment effect. However, RECIST criteria have several limitations for the determination of efficacy of targeted drug activity. This is shown by the fact that in our study, some of the patients with PD based on new lesions had stable or even reduction in the target lesion's longest diameter. Nevertheless, the RECIST criteria are the best criteria to date and should be used in every clinical trial.

Another issue is that in studies of targeted therapy in advanced thyroid cancer, a substantial proportion of patients (33–50%) had SD of varying duration as best response (32). Given the indolent natural history of these tumors, SD may be considered to be of limited value. Considering this, the determination of the best primary end point remains an issue. Objective RRs do not correlate *per se* with OS. Given the indolent nature of advanced thyroid cancer, PFS and OS are not always preferred primary end points. Therefore, only patients with documented progressive disease should be included in clinical trials, permitting a smaller sample size and a shorter time for follow-up to demonstrate a difference in response to therapy between treatment groups. In addition, sorafenib treatment should only be initiated in patients with progressive disease or significant tumor burden in the presence of RECIST targets. Furthermore, patients with SD at baseline may not benefit from therapy and do not need treatment with targeted agents, with all possible side effects.

In conclusion, sorafenib is generally well tolerated and has demonstrated to be clinically active in the long term in advanced RAI refractory DTC. In order to enhance sorafenib treatment efficacy, further trials need to focus on sequential or combination therapy, as patients eventually become progressive on sorafenib or do not tolerate it. More trials with new targeted agentsare underway and the outcomes of these trials will contribute to a better treatment and understanding of advanced thyroid cancer.

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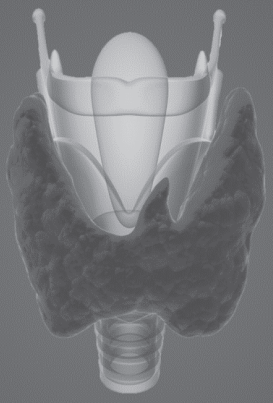
CHAPTER 5

Sorafenib-induced hypothyroidism is associated with increased type 3 deiodination



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Romijn JA
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Smit JW.

J Clin Endocrinol Metab. 2010; 95(8):3758-62

ABSTRACT

Background: Therapy with tyrosine kinase inhibitors is associated with thyroid dysfunction. Decreased serum thyroid hormone levels during tyrosine kinase inhibitors are also observed in athyreotic patients with thyroid carcinoma. We therefore hypothesized that tyrosine kinase inhibitors may influence thyroid hormone metabolism.

Aim: The aim was to study the effects of sorafenib therapy on serum thyroid hormone concentrations and iodothyronine deiodination in athyreotic patients.

Design: This is a retrospective open, single center, single arm 26-weeks phase II study

Methods: We measured serum thyroxine (T4), free T4, 3,5,3-triiodothyronine (T3), free T3, reverse T3 (rT3), and TSH concentrations at baseline and after 26 wk in 21 patients with progressive nonmedullary thyroid carcinoma treated with sorafenib. Ratios of T3/T4 and T3/rT3, which are independent of substrate availability and reflect iodothyronine deiodination, were calculated.

Results: Serum free T4 and T3 levels, adjusted for levothyroxine dose per kilogram body weight, decreased by 11 and 18%, respectively, whereas TSH levels increased. The serum T3/T4 and T3/rT3 ratios decreased by 18 and 22%, respectively, which is compatible with increased type 3 deiodination.

Conclusions: Sorafenib enhances T4 and T3 metabolism, which is probably caused by increased type 3 deiodination.

INTRODUCTION

Therapy with tyrosine kinase inhibitors in patients with malignancies is associated with alterations in thyroid hormone parameters. Sunitinib has been associated with hypothyroidism in 14–85% of the patients, ranging from transient increases in TSH to persistent hypothyroidism, requiring thyroxine substitution (1– 6). In some patients, sunitinib induced hyperthyroidism as well (7–9). Both sunitinib induced hypothyroidism and hyperthyroidism may be caused by destructive thyroiditis, but other mechanisms, such as interference of sunitinib with thyroid peroxidase (4) or inhibition of thyroidal vascularization leading to thyroid atrophy (10, 11), have been proposed. Likewise, sorafenib was associated with TSH elevations in 18% of patients with metastatic renal cell carcinoma (12). All of the above mentioned studies were performed in patients with intact thyroid glands. However, thyroid function abnormalities have also been observed during therapy with imatinib (13, 14), motesanib (15), and sorafenib (16) in athyreotic patients with medullary thyroid carcinoma and nonmedullary thyroid carcinoma. Imatinib increased TSH levels in all patients with medullary thyroid carcinoma (13, 14). Therapy with motesanib caused TSH elevations in 22% of 93 patients with nonmedullary thyroid carcinoma. Sorafenib was associated with increased TSH levels in 33% of thyroid carcinoma patients (16). Because an increased need of thyroxine is observed in athyreotic patients who are treated with tyrosine kinase inhibitors, the mechanisms of hypothyroidism may include alterations in thyroid hormone metabolism. Stepwise deiodination is the major route of thyroid hormone degradation and is mediated by iodothyronine deiodinases (D1, D2, and D3) (17) and by hepatic conjugating enzymes (18). No human studies have been published to address the effect of sorafenib on peripheral thyroid hormone metabolism. We therefore studied the effects of sorafenib, administered for 26 wk, on peripheral thyroid hormone metabolism in athyreotic humans.

PATIENTS AND METHODS

Design

The effects of sorafenib on peripheral thyroid hormone metabolism were analysed in an open, single-center, single-arm 26-wk prospective phase II study, intended to achieve reinduction of Radioiodide uptake in athyreotic patients with progressive metastatic or locally advanced nonmedullary thyroid carcinoma. Results of this study have been published recently (19).

Sorafenib was initially administered at a dose of 400 mg orally twice daily. Exclusion criteria were pregnancy, contraindications for the application of recombinant human TSH (rhTSH), and contraindications for the use of sorafenib. The institutional review board approved the study, and all patients provided written informed consent.

Before initiation of sorafenib therapy and at 26 wk, the patients underwent computed tomography scans for analysis of tumor dimensions and rhTSH (Thyrogen; Genzyme, Naarden, The Netherlands) assisted 4 mCi diagnostic scintigraphies. During the study, patients visited the hospital every month for assessment of thyroid function parameters, biochemical safety parameters, and a physical examination. Fasting morning blood samples were stored at −80 C until analysis.

All patients were treated with levothyroxine, which was adjusted if needed to maintain a target TSH concentration less than 0.1 mU/liter. Only patients who completed 26 wk of treatment with sorafenib were included in the present analysis (per protocol analysis).

Study aims

The objective of this study was to evaluate the effects of sorafenib on thyroid hormone serum levels and to evaluate the effects of sorafenib on iodothyronine deiodinase activity.

Study parameters

The following thyroid function parameters were measured: serum TSH, free thyroxine (fT4), total thyroxine (T4), free 3,5,3-triiodothyronine (fT3), total 3,5,3-triiodothyronine (T3), and reverse 3,5,3-triiodothyronine (rT3). Because levothyroxine dosages were adjusted during the study according to serum TSH levels, serum thyroid hormone levels were corrected for the daily levothyroxine dose and body weight. Dose-adjusted fT4 was calculated as follows: (fT4 (pmol/liter) × weight (kg))/levothyroxine dose (μg).

To assess effects of sorafenib on iodothyronine deiodination, ratios of serum levels of T3/T4and T3/rT3 were calculated. The T3/rT3 ratio is considered to be a sensitive indicator of the peripheral metabolism of thyroid hormone, being positively influenced by D1 and D2 deiodinase and negatively by D3 deiodinase. Serum thyroxine-binding globulin (TBG) levels and fT4/T4 and fT3/T3 ratios were calculated to exclude effects of sorafenib on thyroid hormone binding proteins.

Assays

Fasting blood samples were collected before daily levothyroxine ingestion at both baseline and 26 weeks and stored at −80 C. TSH, fT4, T4, T3, and TBG were measured by chemiluminescence assays (Vitros ECI Immunodiagnostic System; Ortho-Clinical Diagnostics via GE Healthcare, Rochester, NY). fT3 was measured with a RIA (Trinity Biotech, Bray, Ireland). rT3 was measured with an in-house RIA (20). The detection limit of the TSH assay was 0.005 mU/liter. Within-assay coefficients of variation amounted to 4% for TSH, 2% for T4, 2% for T3, and 3–4% for rT3.

Statistics

Data are reported as mean ± SD, median (range), or proportions. Differences in thyroid hormone parameters were analyzed using the two-tailed Student's t test for paired data, comparing measurements between baseline and after 26 weeks sorafenib therapy for normally distributed data, or with a Wilcoxon's test for non-normally distributed data. Differences were considered statistically significant at $P < 0.05$. All calculations were performed using SPSS 16.0 for windows (SPSS, Chicago, IL).

RESULTS

Between October 2007 and October 2008, a total of 32 patients were included in the study. Twenty-two patients completed the 26 weeks because one patient chose not to start therapy and nine patients discontinued treatment during the 26 weeks for disease progression (four patients), adverse events (four patients), and the patient's request (one patient) as reported previously (19). Because the thyroid hormone profile of one patient was not recorded, 21 patients were included in the present analysis: seven females, 14 males, with a median age of 65 years (range, 53–82 years), all with distant metastases of nonmedullary thyroid carcinoma (local, 4%; lungs only, 29%; lungs and bones, 24%; lungs and local, 14%; other, 29%). The efficacy of sorafenib with respect to tumor progression and the presence of adverse effects have been reported previously (19).

Effects of sorafenib on study parameters are given in Table 1. We observed a profound decrease in mean body weight of 6 kg. During the study, the levothyroxine dose per kilogram body weight was significantly increased (2.48 μg/kg before vs. 2.71 μg/kg after sorafenib; P =

0.008) (Table 1, Fig. 1). Nevertheless, TSH increased significantly from 0.051 to 0.545 mU/liter (P = 0.023). Despite the increase in thyroxine dose, significant decreases in serum T3 (1.90 vs. 1.60 nmol/liter) and dose-adjusted free T3 levels (1.59 vs. 1.35 pmol × kg/μg) were observed. Absolute rT3 levels tended to increase after sorafenib therapy, and T3/T4, T3/rT3, and T4/rT3 ratios decreased significantly by 18, 22, and 7%, respectively, reflecting an increased conversion of T4 into rT3. Potential effects of sorafenib on thyroid hormone binding proteins were assessed by measuring TBG levels, which were not influenced by sorafenib, and by calculating ratios of free over total T4 and T3 concentrations, which did not differ before and after sorafenib.

Table 1 Thyroid Hormone Parameters

	N	Baseline	26-wks sorafenib	P –value
Weight (kg)	21	77.3 ± 13.5	71.0 ± 14.2	<0.001
Sorafenib dose (mg/kg.d)	21	10.4 ± 2.4	8.4 ± 2.7	0.003
Levothyroxine dose (μg/kg.d)	21	2.48 ± 0.67	2.71 ± 0.61	0.008
T4 (nmol/L)	21	149.4 ± 31.9	152.9 ± 37.7	0.664
T3 (nmol/L)	20	1.90 ± 0.33	1.60 ± 0.34	0.007
fT4 (pmol/L)	21	27.3 ± 5.9	26.6 ± 6.9	0.587
fT4 (pmol/L) × weight (kg)/ levothyroxine dose (μg)	21	11.7 ± 3.6	10.4 ± 3.6	0.034
fT3 (pmol/L)	13	5.43 ± 0.71	4.80 ± 1.09	0.075
fT3 (pmol/L) × weight (kg)/ levothyroxine dose (μg)	13	1.59 ± 1.38	1.35 ± 1.15	0.005
rT3 (nmol/L)	20	0.68 (0.50- 1.02)	0.79 (0.63- 1.12)	0.091[a]
TSH (mU/L)	21	0.01 (0.005- 0.33)	0.100 (0.005- 4.670)	0.023[a]
TBG (mg/L)	21	19.8± 4.3	19.5 ± 4.0	0.620
fT3/T3 $\times 10^3$	13	2.73 ± 0.33	2.93 ± 0.31	0.279
fT4/T4 $\times 10^3$	21	5.55 ± 0.94	5.84 ± 0.95	0.124
T3/T4 × 100	20	1.28 ± 0.00	1.05 ± 0.00	<0.001
T3/rT3	20	2.74 ± 0.50	2.16 ± 0.53	<0.001
T3/rT3	20	220 ± 27	205 ± 32	0.036

Data expressed as mean ± SD or median (range)
[a] Wilcoxon`s Test.

DISCUSSION

In this study, we assessed the relationship between treatment with the multiple target kinase inhibitor sorafenib and alterations in thyroid hormone parameters. Therapy with tyrosine kinase inhibitors is associated with hypothyroidism. Proposed mechanisms include direct effects of these drugs on the thyroid, including destructive thyroiditis (4, 10, 11). Because

thyroid function abnormalities have also been observed in athyreotic patients on thyroxine substitution, the mechanisms of hypothyroidism may include alterations in thyroid hormone metabolism as well. We hypothesized that sorafenib may influence the activities of iodothyronine deiodinases (D1, D2, and D3) (17), which has not been studied in humans so far. We found that a higher substitution dose of thyroxine was needed to maintain serum fT4 levels. These findings are consistent with a previous report in which eight imatinib treated patients that had undergone thyroidectomy required substantial increases in levothyroxine replacement dose (13). Indeed, the increased need of levothyroxine may be underestimated because some patients may have been over treated before initiation of the trial. In addition, we found a clear decrease in serum T3/T4, T3/rT3, and T4/rT3 ratios. These ratios reflect alterations in the peripheral metabolism of thyroid hormone, being positively influenced by deiodinases D1 and D2 and negatively by D3 (17). Moreover, they are independent of levothyroxine administration. The decreased T3/T4 and T3/rT3 ratios may be caused by a decrease in D1 and/or D2 activity. However, this would be associated with a decreased rather than an increased T4 metabolism. Therefore, the decreased T3/T4 and T3/rT3 ratios are best explained by an increased D3 activity.

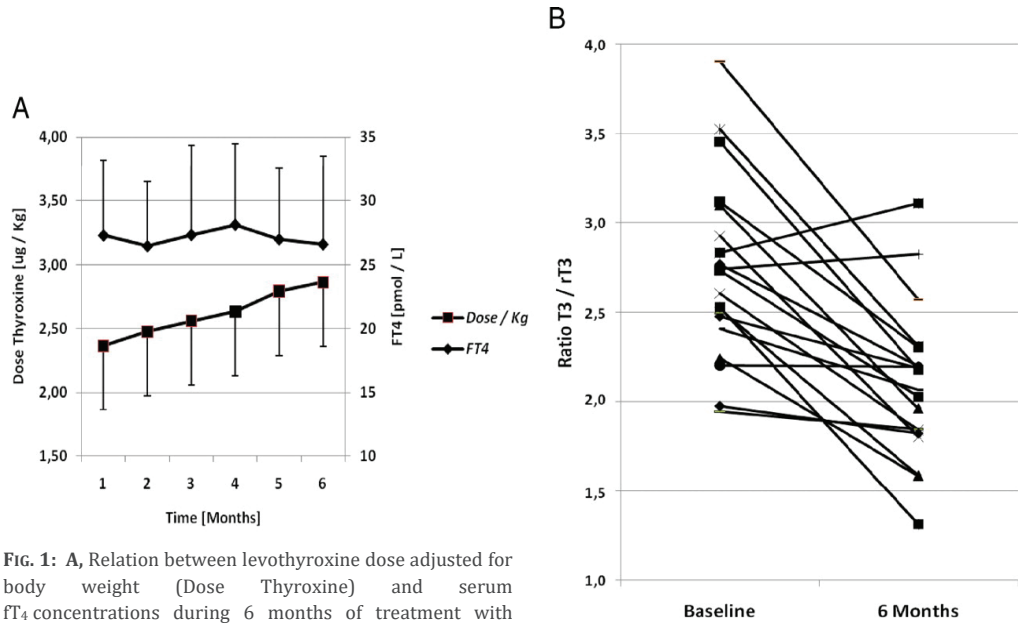

FIG. 1: A, Relation between levothyroxine dose adjusted for body weight (Dose Thyroxine) and serum fT_4 concentrations during 6 months of treatment with sorafenib. **B,** Individual T_3/rT_3 ratios before and 6 months after treatment with sorafenib.

The fact that, 2 months after the start of sorafenib, a significantly higher T4 dose per kilogram body weight was necessary to maintain fT4 concentrations strongly suggests that the phenomenon is already present early after initiation of sorafenib therapy. It is worthwhile to further elucidate the effects of sorafenib on D3 in in vitro studies. It is unlikely that the increased D3 activity reflects a state of nonthyroidal illness because serum TSH increased during sorafenib, whereas in nonthyroidal illness, decreased rather than increased TSH levels would be expected. Although it can be hypothesized that decreased absorption of T4 could also have played a role, the interval between sorafenib and thyroxine intake was approximately 12 h. In addition, decreased T4 absorption would not affect T3/T4 and T3/rT3 ratios. No changes in TBG levels and the ratios between free and bound thyroid hormones were observed, ruling out effects of sorafenib on thyroid hormone binding proteins, which again, even if present, would not have affected the T3/rT3 ratio. It may hypothesized that sorafenib may also influence conjugation of thyroid hormone with glucuronates and sulfates by hepatic microsomal enzymes. However, altered conjugation would not influence T3/rT3 ratios. The fact that the increase in overall rT3 levels was non-significant may suggest that rT3 degradation may be enhanced as well. It is likely that, because rT3 is the preferred substrate for type1 deiodinase (in the absence of hyperthyroidism), increased rT3 may lead to enhanced type 1 deiodinase-mediated rT3 degradation.

In conclusion, this study shows that, in addition to direct effects of tyrosine kinase inhibitors on the thyroid gland, enhanced peripheral metabolism of thyroid hormone, likely by activity of type 3 deiodinase, may contribute to hypothyroidism during therapy with these drugs.

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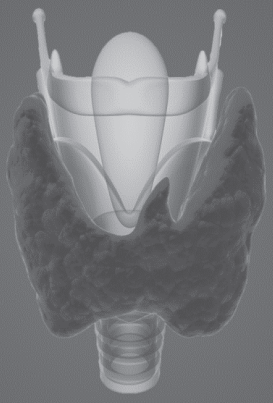
CHAPTER 6

Impact of Metformin and compound C on NIS expression and iodide uptake in vitro and in vivo: a role for CRE in AMPK modulation of thyroid function



Abdulrahman RM
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Thyroid. 2014; 24(1):78-87

ABSTRACT

Background: Although adenosine monophosphate activated protein kinase (AMPK) plays a crucial role in energy metabolism, a direct effect of AMPK modulation on thyroid function has only recently been reported, and much of its function in the thyroid is currently unknown. The aim of this study was to investigate the mechanism of AMPK modulation in iodide uptake. Furthermore, we wanted to investigate the potential of the AMPK inhibitor compound C as an enhancer of iodide uptake by thyrocytes.

Methods: The in vitro and in vivo effects of AMPK modulation on sodium-iodide symporter (NIS) protein levels and iodide uptake were examined in follicular rat thyroid cell-line cells and C57Bl6/J mice. Activation of AMPK by metformin resulted in a strong reduction of iodide uptake (up to sixfold with 5mM metformin after 96 h) and NIS protein levels in vitro, whereas AMPK inhibition by compound C not only stimulated iodide uptake but also enhanced NIS protein levels both in vitro (up to sevenfold with 1 μM compound C after 96 h) and in vivo (1.5-fold after daily injections with 20mg/kg for 4 days). We investigated the regulation of NIS expression by AMPK using a range of promoter constructs consisting of either the NIS promoter or isolated CRE (cAMP response element) and NF-κB elements, which are present within the NIS promoter.

Results: Metformin reduced NIS promoter activity (0.6-fold of control), whereas compound C stimulated its activity (3.4-fold) after 4 days. This largely coincides with CRE activation (0.6- and 3.0-fold). These experiments show that AMPK exerts its effects on iodide uptake, at least partly, through the CRE element in the NIS promoter. Furthermore, we have used AMPK-alpha1 knockout mice to determine the long-term effects of AMPK inhibition without chemical compounds. These mice have a less active thyroid, as shown by reduced colloid volume and reduced responsiveness to thyrotropin.

Conclusion: NIS expression and iodide uptake in thyrocytes can be modulated by metformin and compound C. These compounds exert their effect by modulation of AMPK, which, in turn, regulates the activation of the CRE element in the NIS promoter. Overall, this suggests that the use of AMPK modulating compounds may be useful for the enhancement of iodide uptake by thyrocytes, which could be useful for the treatment of thyroid cancer patients with radioactive iodide.

INTRODUCTION

Adenosine monophosphate-activated protein kinase (AMPK) is an energy-sensing heterotrimeric complex that plays an important role in the energy balance at both the cellular level and the whole body by balancing nutrient supply and demand. It consists of a catalytic α-subunit and two regulatory subunits, β and γ, which in turn are expressed as different isoforms (α1, α2, β1, β2, γ1, γ2 and γ3). Theoretically, this enables the formation of 12 different complexes that may reflect the function of AMPK through different subcellular locations and signalling functions enabling the activation of different pathways (1,2). AMPK is known to influence the function of a variety of organs, such as the liver and pancreas (3). However, reports on the role of AMPK in thyroid function and iodide uptake remain scarce, and much of its function in the thyroid is currently unknown. AMPK can be activated by a range of stimuli, for example hypoxia, starvation, and glucose deprivation, which leads to the activation or inhibition of multiple downstream pathways (4,5). Different compounds are known to modulate AMPK activity, such as the AMPK activators metformin and 5-aminoimidazole-4-carboxamide-1-b-D-ribofuranoside (AICAR), and the AMPK inhibitor compound C, which is a small-molecule cell-permeable pyrrazolopyrimidine derivative that acts as a reversible ATP competitive inhibitor of AMPK (6–8).

In the normally functioning thyroid, iodide accumulation is an essential step in the production of thyroid hormone (TH) and is mediated by the sodium iodide symporter (NIS). NIS expression itself is driven by several thyroid specific and non-specific regulators (9-11). Furthermore, iodide uptake is also regulated by the presence or absence of NIS on the cell surface. The shuttling of NIS between cell-surface and cytoplasm provides an extra layer of regulation and is dependent on several factors with an emphasis on thyroid stimulating hormone (TSH) stimulation (11-13). The accumulation of iodide by thyrocytes is also exploited in the treatment of thyroid carcinomas by the administration of radioactive iodide-131 (^{131}I; RAI-therapy) and is often the only curative option for recurrent or metastatic disease (14-16).

Although both AMPK and the thyroid play a crucial role in energy metabolism (3,17), a direct effect of AMPK modulation on thyroid function has only recently been reported in a study by Andrade et al. (18). In this study, iodide uptake and NIS expression were suppressed after treatment with the AMPK agonist AICAR in PCCL3 follicular thyroid cells, an effect

which could be reversed by the AMPK inhibitor Compound C. Furthermore, iodide uptake was reduced in rat thyroid glands after 24 h following a single injection with AICAR.

The aim of our study was to further investigate the role of AMPK in the thyroid using the AMPK activator Metformin and the AMPK inhibitor Compound C, and to provide mechanistic insights into its role in NIS expression and iodide uptake. Our findings give new insight in the subunit composition of AMPK and demonstrate the effects of prolonged modulation of AMPK, an aspect that could be of great importance in the treatment of thyroid cancer, which depends on NIS expression and RAI uptake (14-16). We show for the first time that inhibition of AMPK by Compound C in the thyroid results in increased NIS expression, iodide uptake and plasma T4 levels. Thus, inhibition of AMPK or one of its downstream pathways may be a promising treatment strategy to enhance iodide uptake by thyrocytes, which might enhance RAI treatment of thyroid cancer patients. Furthermore, we provide insight in the regulation of *NIS* expression by AMPK using a range of promoter constructs consisting of either the *NIS* promoter or isolated CRE (cAMP response element) and NF-κB elements, which are present within the *NIS* promoter. These experiments show that AMPK exerts its effects on iodide uptake, at least partly, through the CRE element in the *NIS* promoter.

MATERIALS AND METHODS

Cell culture

The follicular rat thyroid cell-line (FRTL-5) was purchased from Health Protection Agency Culture Collections (Salisbury, UK) (19). FRTL-5 cells were routinely cultured in Coon's modified Ham's F-12 medium supplemented with 10% charcoal stripped calf serum and a six-hormone (6H) preparation consisting of 5 hormones (5H) (10 μg/mL human recombinant insulin (Sigma), 10 ng/mL somatostatin (Sigma), 10 nM hydrocortisone (Sigma), 5 μg/mL transferrin (Sigma) and 10 ng/mL glycyl-L-histidyl-L-lysine acetate (Sigma) supplemented with 100 mU/L bovine TSH (Sigma) (making 6H medium). Cells were maintained in a 5% CO_2-95% air atmosphere at 37°C with a change of medium every third day, and passed every 7 days. Prior to incubation with AMPK modulating chemicals, monolayers were cultured without calf serum for 8 h.

***In vitro* treatments**

FRTL-5 cells were treated with Metformin or Compound C at a concentration of respectively 2 mM and 5 μM for *in vitro* work.

Iodide uptake in FRTL-5 cells

Iodide uptake was determined as described previously (20,21). Briefly, 24 h before the assay, cells were washed and cultured in serum-free medium containing 6H or 5H for TSH-induced iodide uptake in time (19). After washing twice with Hanks balanced salt solution (HBSS), cells were incubated for 30 min with 500 μL of HBSS containing 0.1 μCi of $Na^{125}I$ and 20 μM unlabeled NaI, with or without 80 μM of sodium perchlorate to control for specific uptake. The radioactive medium was aspirated; cells were washed twice with 1 mL of ice-cold HBSS and accumulated iodide was extracted with 1 mL of 1xSSC+ 0.1% SDS for 30 minutes. All fluids were counted in a γ-counter. Accumulated radioactivity was expressed as pmol NaI/μg DNA.

Western blot

FRTL-5 cells were lysed directly in Laemmli sample buffer (BioRad, Hercules, CA, USA) and thyroid tissue extracts were prepared in NP buffer using a mini bead beater (BioSpec, Bartlesville, OK, USA) with 1 mm glass beads (Sartorius AG, Goettingen, Germany). Thyroid tissue samples obtained from *in vivo* studies were subsequently diluted 1:1 in Laemmli buffer. Cell or thyroid tissue extracts were subjected to SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (0.45 μm) (Pierce, Rockford, IL, USA) all subsequent steps were performed in the presence of a commercial protease inhibitor cocktail in the recommended dose (Roche, Basel Switzerland). After 20 min of blocking at room temperature, the membrane was incubated (1:1000) with the primary antibody GAPDH (sc-25778) (Santa Cruz Biotechnology, Santa Cruz, Ca, USA) at 4°C O/N followed by an 1 h incubation with horseradish peroxidase conjugated secondary antibody. The immune complexes were visualized using the Molecular Imager ChemiDoc XRS System (Biorad, Hercules, CA, USA). Following equalization of the amounts of protein by comparing GAPDH expression, the following primary antibodies and dilutions were used: NIS (1:1000), a kind gift of N. Carrasco, AMPKα1 (1:2500), AMPKα2 (1:2500) (Kinasource, Dundee, UK), AMPKβ 1+2 (1:1000), AMPKγ2 (1:1000), p-ACC(Ser79) (1:1000) (Cell Signaling Technology, Inc., Beverly, MA, USA), AMPKγ1 (1:500), and AMPKγ3 (1:500) (Santa Cruz

Biotechnology, Santa Cruz, Ca, USA). NIS protein expression of the *in vivo* samples was quantified using the Molecular Imager ChemiDoc XRS System (Biorad, Hercules, CA, USA)

Luciferase promoter reporter assays

To study transcriptional regulation of *NIS* expression by AMPK modulation, we have obtained a range of promoter-luciferase constructs which either contain all the known functional regions of the NIS upstream enhancer region (NUE; including CRE and NF-κB) plus the proximal promoter of the rat *NIS* promoter or an isolated CRE or NF-kb element. FRTL-5 cells were transiently transfected immediately after seeding in 5H medium with either rNIS9-LUC [containing the proximal promoter – 1 to - 564 bp (relative to ATG) and the NUE region - 2264 bp to - 2495 bp (relative to ATG) in pGL3 (10)], CRE-LUC (kindlyprovided by A. Himmler, Ernst Boehringer Institute, Bender + Co. GmbH, Vienna, Austria) or NF-κB -LUC (Stratagene, La Jolla, Ca, USA). Renilla luciferase (pRL-CMV; Promega, Madison, WI, USA) was co-transfected to correct for transfection efficiency. After 24 h, the medium was replaced by fresh 5H or 6H medium with or without the addition of Metformin or Compound C. Promoter activity (luciferase/renilla) is depicted as % of controls.

Animals

For all *in vivo* experiments with AMPK modulating compounds, 8-week old male C57Bl6/J mice were used. Mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed in conventional cages with a 12:12-h light-dark cycle and free access to food and water. All animal experiments were approved by the institutional ethics committee on animal care and experimentation at Leiden University Medical Center (Leiden, the Netherlands).

of thyroids from AMPK-alpha 1 nockout mice (22) and their littermates were a kind gift from Dr. Benoit Viollet (Département endocrinologie, métabolisme et cancer, Inserm, Institut Cochin Paris, France) and Dr. Sandrine Horman (Université catholique de Louvain, Brussels, Belgium).

***In vivo* treatments**

To evaluate the effect of AMPK modulation on NIS expression *in vivo,* mice were randomized according to their body weight and divided into 3 groups (n=9). Mice received daily i.p. injections with either Metformin (100 mg/kg), Compound C (20 mg/kg) or vehicle (PBS; control) for 4 days while feeding on a chow diet.

***In vivo* iodide uptake**

After 4 days of treatment with Metformin, Compound C or vehicle, the *in vivo* iodide uptake was determined. Mice were injected with 200 µL of $Na^{125}I$ ($1.0x10^6$ cpm; Perkin Elmer, Waltham, MA, USA) via the tail vein. After 15 min, mice were sacrificed via cervical dislocation and perfused with ice-cold PBS through the right ventricle to wash out residual ^{125}I. The thyroids were subsequently taken out and ^{125}I-activity was counted using a γ-counter.

Plasma T4 levels

Plasma T4 levels were measured using the 1100 T4 ELISA kit (Alpha diagnostic, San Antonio, TX, USA) and TSH levels were measured using the E90463Mu TSH ELISA kit (USCN Life Science Inc., Houston, TX, USA) according to the manufacturer's instructions.

Immunohistochemistry

Mouse thyroids were embedded in paraffin and cut into 4-5 µm sections. The sections were deparaffinized in histoclear for 10 min. Endogenous peroxidase was blocked with hydrogen peroxide (0.3% H_2O_2 in methanol) at room temperature for 20 min. After rehydration, antigen was retrieved by boiling for 10 min in 0.1 M citric acid, pH 6.0. After cooling to room temperature and washing with PBS, slides were incubated overnight with primary antibody (1:1000) in 1% BSA at 4°C. The next day, slides were washed and incubated for 30 min at room temperature with HRP-labeled polymer anti-rabbit (DAKO envision system) (Dako, Glostrup, Denmark) followed by an incubation with the peroxidase substrate, vector nova red (Vector Laboratories, Burlingame, CA, USA) for 5 min. The reaction was stopped in milliQ and the sections were counterstained with Mayer's hematoxylin, washed, dehydrated and mounted in histomount. The slides were subsequently visualized under a microscope (Eclipse E800, NIKON instruments inc., Melville, NY, U.S.A.)

Statistical analyses

Results are expressed as means ± the standard error of means. All data have been analyzed by one-way analysis of variance (ANOVA) followed by a Tukey's post-hoc test with the exception of the results shown in Figure 1 (two-way ANOVA) and Figure 8 (Student's t-test). All statistical analyses were performed using SPSS v14.0 (SPSS, Inc., Chicago, IL)

RESULTS

Metformin and Compound C influence NIS expression and iodide uptake in vitro

First, we investigated the effect of AMPK activation and inhibition on NIS expression and iodide uptake *in vitro* by treating follicular thyroid cells (FRTL-5 cells) with Metformin or Compound C, respectively. The AMPK activator Metformin suppressed TSH-induced iodide uptake in a time- (significant at 16 h) and dose-dependent manner which coincided with a reduction in NIS protein levels (Fig. 1, 2). Treatment of Metformin-stimulated FRTL-5 cells with Compound C, an AMPK inhibitor, counteracted the effects of Metformin on iodide uptake and NIS expression (Fig. 3). Furthermore, Compound C treatment alone was able to increase iodide uptake (significant at 96 h) up to 7-fold after 4 days of treatment (Fig. 1, 3). This increase in iodide uptake coincided with an increase in NIS protein. Interestingly, as shown in Fig. 3B, in the absence of TSH, remnant NIS protein levels could be repressed even further by Metformin or enhanced by Compound C (Fig. 3B).

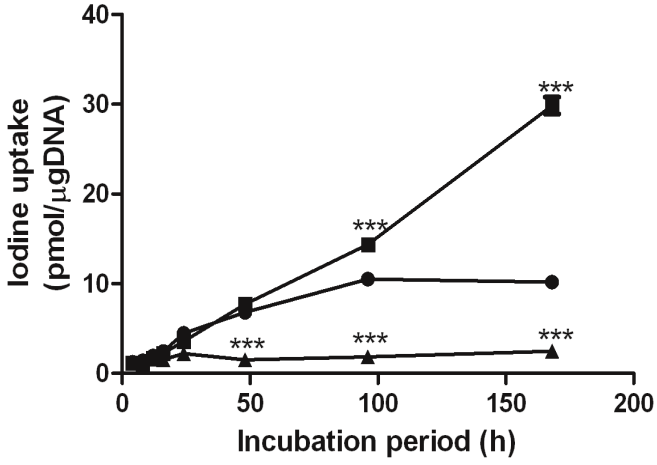

Fig.1

Figure.1. Effect of Metformin and Compound C on TSH-induced iodide uptake by FRTL-5 cells. TSH-induced iodide uptake in FRTL-5 cells (●) is altered by Metformin (▲) and Compound C (■) in a time-dependent manner. Values are means ± SD (n=3). *** p<0.001 vs. control

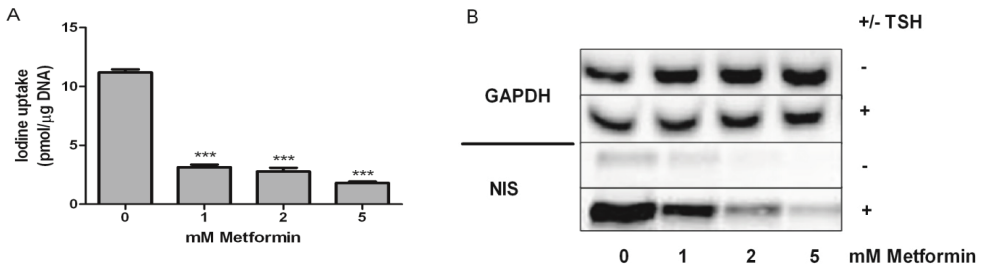

Figure. 2**.** Dose-dependent effect of metformin treatment on TSH-induced iodide uptake and sodium-iodide symporter (NIS) protein levels in FRTL-5 cells after 96 h of treatment. Iodide uptake was corrected for DNA content **(A)**, and NIS protein levels were equilibrated for GAPDH (Western blot) **(B)** in stimulated FRTL-5 cells (+ TSH). Values are mean ± SD (n = 3). ***p < 0.001 vs. control.

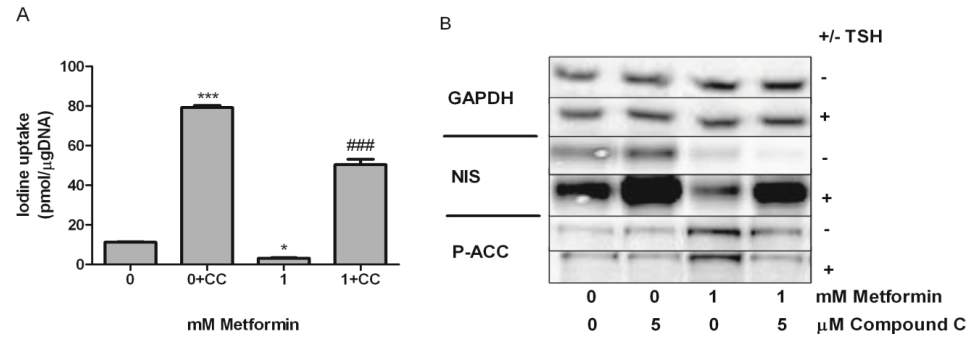

FIG. 3. Effect of compound C (CC) on TSH-induced iodide uptake and NIS protein levels in FRTL-5 cells. Compound C treatment (AMPK inhibitor) increased iodide uptake and counteracted the effects of metformin (AMPK activator) on iodide uptake **(A)**. The observed effects on iodide uptake coincide with NIS protein levels, as shown by Western blot **(B)** in stimulated FRTL-5 cells (+ TSH) after 96 h. Acetyl-CoA carboxylase (ACC) that is directly phosphorylated by AMPK functions as an AMPK activation control. Values are mean ± SD (n = 3). ***p < 0.001 vs. control; ###p < 0.001 vs. corresponding metformin dose.

AMPK α1, β1 and γ1 are the predominant subunits in FRTL-5 cells

As the expression patterns of the different isoforms of AMPK (α1, α2, β1, β2, γ1, γ 2 and γ3) in thyrocytes are unknown, we determined the expression level of each of the subunits by western blot in FRTL-5 cells. We discovered that the predominant subunits of AMPK are α1, β1 and γ1. Moreover, treatment of the FRTL-5 cells with either Metformin or Compound C, which are known to modulate AMPK activation by phosphorylation of the α -subunit at Thr-172, did not alter the predominance of α1, β1 and γ1 over the other isoforms (Fig. 4).

Regulation of NIS transcription by Metformin and Compound C

The decrease in iodide uptake after Metformin treatment or the increase after Compound C treatment could, at least partially, be explained by de- or increased NIS protein levels (Fig. 2, 3). This prompted us to investigate the transcriptional regulation of NIS by Metformin and Compound C. The regulatory elements in the *NIS* promoter are located in the *NIS* upstream enhancer region (NUE), which includes CRE and NF-κB elements. At day 1, Metformin did not inhibit the activation of any of the promoter constructs. However, Compound C was able to increase CRE promoter activity 4.8 fold and slightly activated both rNIS and NF-kb promoter activity by respectively 1.2 and 1.6 fold (Fig 5A). At day 4, activation of the CRE element coincided with *rNIS*-promoteractivity after treatment with either Compound C or Metformin (Fig. 5B). Metformin treatment resulted in a decrease in both *rNIS* and CRE reporter activity of respectively 1.8 fold and 1.9 fold. Compound C treatment resulted in an increase in *rNIS* and CRE reporter activity of respectively 3.4 and 3.0 fold.

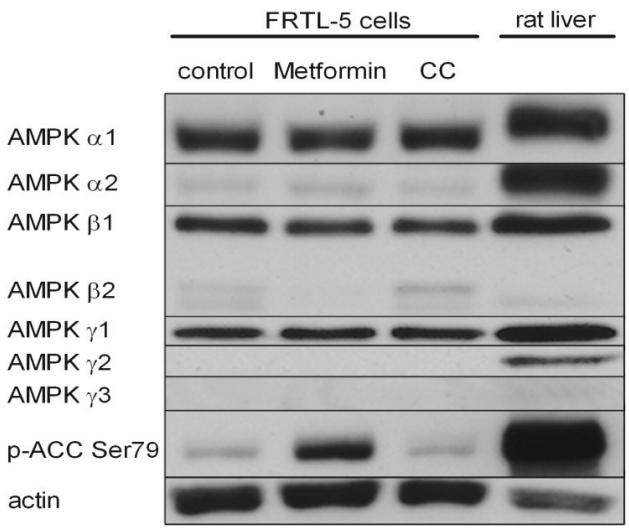

FIG. 4. Prevalence of AMPK subunits in FRTL-5 cells.FRTL-5 cells were treated for 4 days with metformin or compound C. The amount of protein was visualized by Western blot after normalization for actin. ACC-ser79 phosphorylation (p-ACC-Ser79) was used as a measure for AMPK activation with rat liver tissue as a positive control.

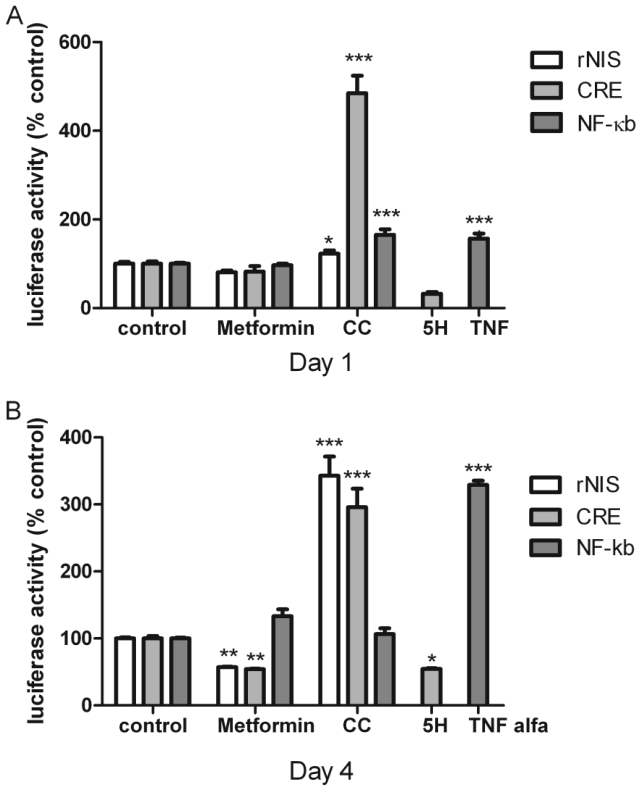

FIG. 5. Activation of reporter constructs after 1 day **(A)** and 4 days (**B**) of metformin or compound C treatment.
The luciferase-reporter constructs include the rat NIS promoter (fusion of the rNIS proximal promoter -1 to - 564 bp and the NUE region - 2264bp to - 2495 bp, which contains a CRE element and an NF-κB element) an isolated 3 CRE element and an isolated 3· NF-κB element. 5H medium (lacking TSH) was used as a negative control for CRE activation and TNFα was used as a positive control for NF-κB. Values are mean ± SD (n = 6). *p < 0.05 vs. control; **p < 0.01 vs. control; ***p < 0.001 vs. control.

Regulation of thyroid function in vivo

To investigate whether modulation of AMPK activity could also influence NIS expression and iodide uptake in thyroid *in vivo*, we treated mice daily for 4 days. Mice received daily i.p. injections with either Metformin (100 mg/kg), Compound C (20 mg/kg) or vehicle (PBS; control) for 4 days. After 4 days of treatment body weight was not affected by either treatment although initially the Compound C treated group had a slightly decreased body weight at day 2 of 8% ($p<0.05$). Although Metformin treatment reduced immunohistochemical staining of NIS protein in the thyroid, the decrease in NIS protein levels was nonsignificant and ^{125}I uptake was unaffected (Fig 6A-C). This indicates that the effect of Metformin on NIS protein levels was insufficient to reduce iodide uptake.

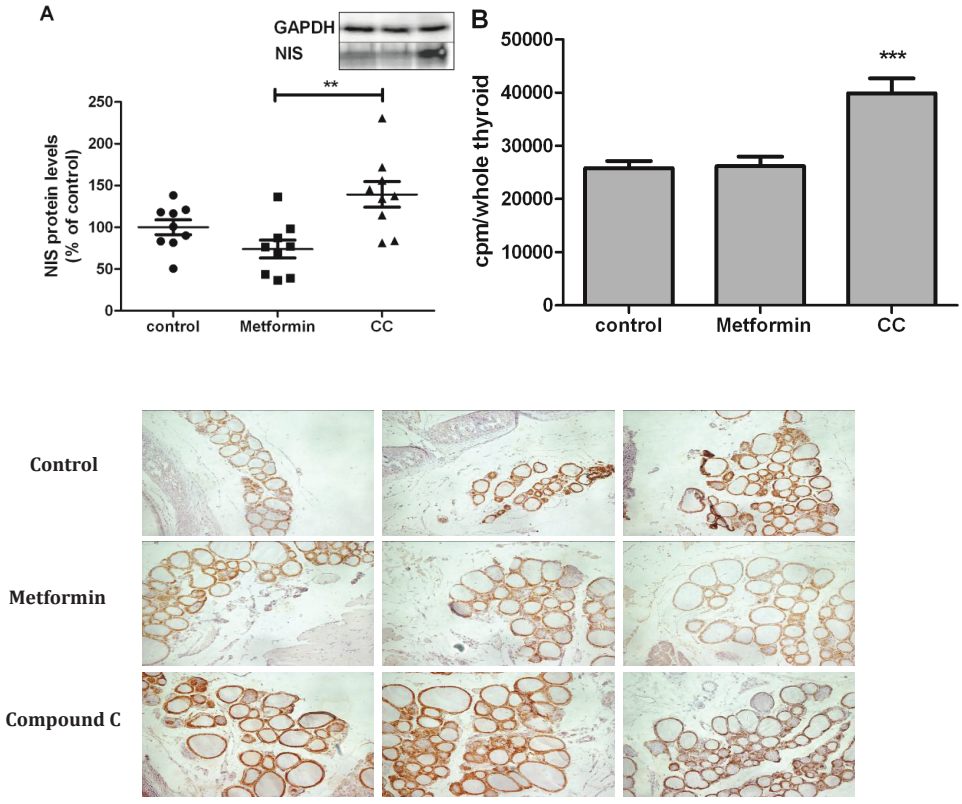

FIG. 6. NIS protein levels and iodide uptake in mice after 4 days of treatment with metformin or compound C. Mice received daily i.p. injections with metformin (100 mg/kg), Compound C (20 mg/kg) or vehicle for 4 days. NIS protein levels in thyrocytes were analyzed by Western blot (A) (n = 9/group) and IHC staining (C) (n = 3/group). NIS Western blot data of individual animals were quantified and corrected for GAPDH (A) (inlay pooled fractions) and NIS protein levels were depicted as % of controls ([NIS/GAPDH]/controls · 100%). Iodide uptake (B) is depicted as cpm/whole thyroid. Values are mean – SD (n = 8). **p < 0.05 vs. control; ***p < 0.001 vs. control.

However, after Metformin treatment T4 levels were significantly lower than in Compound C treated animals (Fig. 7A) but no effects on TSH levels were observed (Fig. 7B). A possible explanation for the apparent discrepancy between NIS function and T4 levels could be that Metformin-treated animals were slightly ill (23), or it may be due to a reduction in serum T4 binding proteins which are known to be regulated by metabolic parameters (24). In line with our *in vitro* data, treatment of mice with Compound C resulted in marked upregulation of NIS protein levels (+39%, $p<0.05$) and ^{125}I uptake (+55%, $p<0.001$) in thyroid tissue (Fig 6A, B). This was also supported by the finding of increased immunohistochemical staining of NIS protein (Fig 6C).

The effects of AMPK-alpha 1 on thyroid function were studied in AMPK alpha 1 knockout mice (Fig. 8). When compared to controls these mice have an increased colloid diameter (Fig. 8A,B), raised TSH levels (6-fold) (Fig. 8C) and decreased T4 levels (0.6-fold) while T3 levels were unaffected (Fig. 8 D, E).

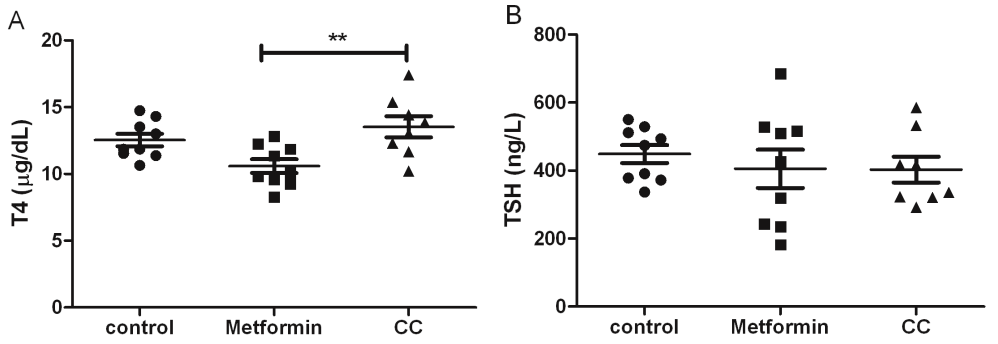

FIG. 7. T4 and TSH serum levels after 4 days of treatment. Mice received daily i.p. injections with metformin (100mg/kg), compound C (20 mg/kg), or vehicle for 4 days. T4 **(A)** and TSH **(B)** serum levels were determined by ELISA (n = 9/group). **p < 0.05 vs. control.

DISCUSSION

The aim of this study was to investigate the mechanism of AMPK modulation of iodide uptake in thyrocytes. Furthermore, we evaluated the potential of the AMPK inhibitor Compound C as an enhancer of iodide uptake by thyrocytes, which could be of potential benefit in the RAI treatment of thyroid cancer patients. We show that NIS expression and iodide uptake in thyrocytes can be upregulated by Compound C, *in vitro* and *in vivo*. Furthermore, we show that AMPK exerts its effects on iodide uptake, at least partly, through stimulation of the CRE element in the *NIS* promoter.

In vitro, Metformin was able to increase AMPK activity, which resulted in a strong reduction of iodide uptake and NIS expression, whereas treatment with the AMPK-inhibitory Compound C resulted in a strong increase of iodide uptake and NIS expression. Although these chemical compounds may induce cellular effects which are not directly linked to AMPK repression/or stimulation (25-29) the opposing effects of Metformin and Compound C on iodide uptake and NIS expression point towards an AMPK-related mechanism, which is further supported by a recent study where AICAR was able to inhibit iodide uptake in PCCL3 follicular thyroid cells and rat thyroid glands (18). In addition, we demonstrate that AMPK in

FRTL-5 cells predominantly consists of the subunits α1, β1 and γ1 (30). The subunit composition of AMPK may reflect AMPK function in the thyroid (1,2), although this is still under debate.

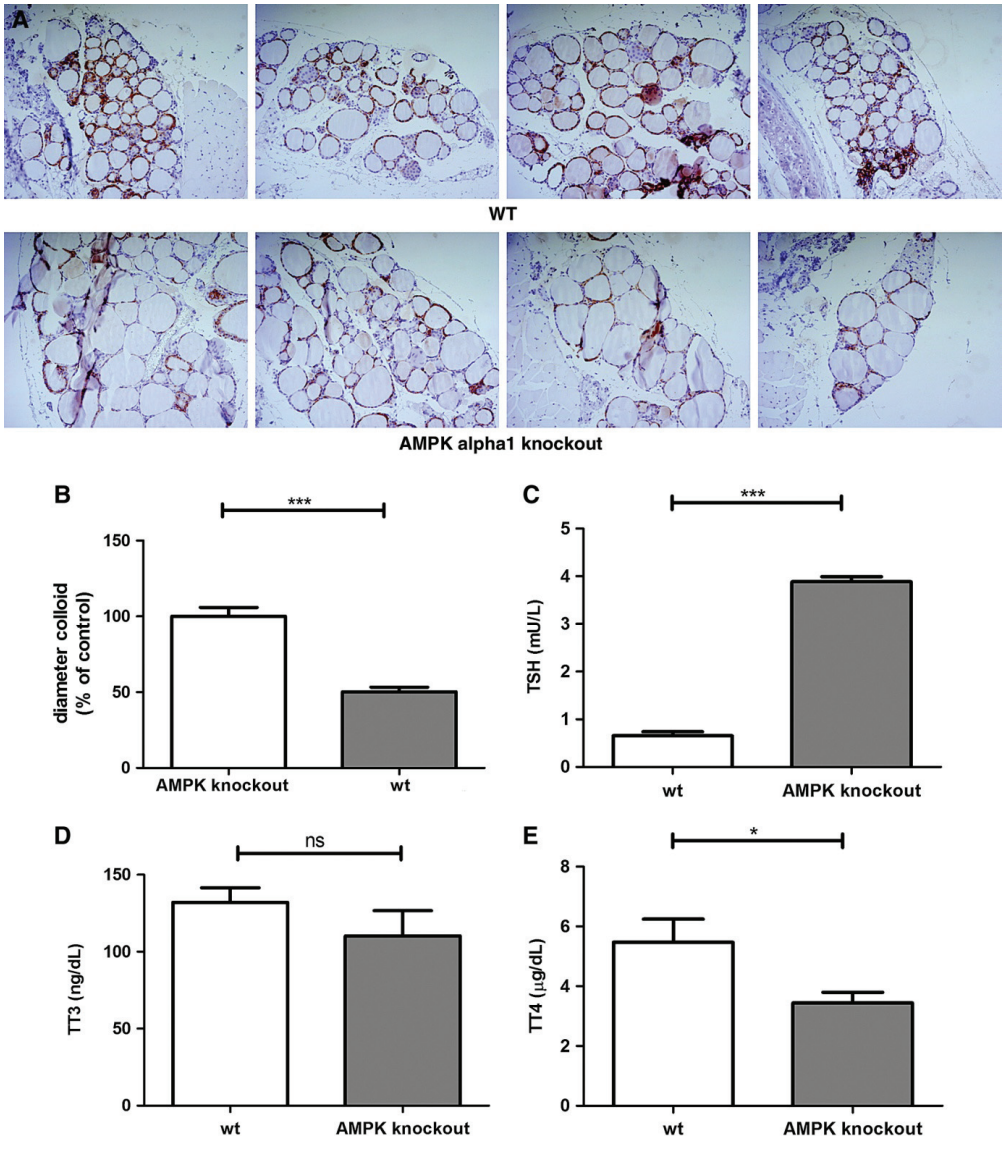

FIG. 8. Difference in follicle size **(A, B)** and TSH **(C)**, T3 **(D)**, and T4 **(E)** serum levels between wild type and AMPK-alpha 1 knockoutmice. TSH**(C)**, T3**(D)**, andT4**(E)** serum levelswere determined by ELISA (n = 4/group). *p < 0.05 vs.wt; ***p < 0.001 vs.wt.

In vivo, Compound C clearly enhanced iodide uptake which suggests that AMPK inhibition may provide a useful mechanism for the enhancement of iodide uptake which may be of potential benefit in the treatment of papillary and follicular thyroid carcinomas with RAI (14-16). A possible use of AMPK inhibiting compounds in the enhancement of RAI therapy will require further investigation. It should be noted that AMPK modulation is known to modulate several factors involved in growth; for example, the glucose transporter protein GLUT 1 e.g. Glut 1 is up-regulated by AICAR at a concentration of 1,5 mM after 24h resulting in reduced glucose uptake in the follicular thyroid cell-line PCCL3 this effect could be reversed by Compound C (30). Furthermore, it is unclear if inhibition or stimulation of AMPK will inhibit or increase growth of tumor cells as both have been documented to suppress growth (31,32).

Considering the fact that at this point it is unclear what the effects of AMPK modulation on growth and iodide uptake will be in thyroid tumors, we can only speculate on the clinical impact of AMPK modulation on iodide uptake. However, we think that unraveling the pathways downstream of AMPK leading towards enhanced iodide uptake may enable the differentiation between enhanced iodide uptake and effects on growth.

As the uptake of iodide is the first essential step in the production of TH, we also examined serum T4 levels. There was no difference between control and either treatment but T4 levels were significantly higher in the Compound C treated group when compared to the Metformin treated group (28% increase). Remarkably, TSH levels remained unaffected.

We are aware that long term Metformin treatment (12 months) has been associated with a reduction in TSH levels (33-35). These studies describe a lowering of serum TSH levels in hypothyroid and euthyroid diabetic patients with basal TSH levels in the upper normal range without affecting T4 levels. So far no definite explanation has been given for these observations but in our view a possible explanation may be that Metformin sensitizes the thyroid for TSH stimulation after a few months of treatment.

Our study describes the shortterm effects of AMPK modulation by Metformin and Compound C treatments. 4 days of Metformin treatment resulted in decreased iodide uptake and NIS protein levels in follicular thyroid cells, which would suggest a decrease in thyroid function. Therefore short- (4 days) and long-term (1 year) stimulation of AMPK by Metformin seem to have an opposite effect on thyroid function. This is further supported by

the addition of new data from AMPK alpha knockout mice, which have increased serum TSH levels without changes in T4 levels (completely opposite to long term AMPK stimulation by Metformin) (33-35).

The *in vitro* decrease in iodide uptake after Metformin treatment or the increase after Compound C treatment can, at least partially, be explained by decreased or increased NIS protein levels (Fig. 2 and 3). We therefore decided to analyze the transcriptional regulation of the *NIS* promoter. The main regulatory region in the *NIS* promoter is the *NIS* upstream enhancer (NUE) region which contains response elements for TTF-1, CRE, Pax8 and NF-□□ (9-11, 36). The *rNIS* promoter containing both the NUE region and the proximal promoter were activated after 4 days of Compound C treatment, whereas Metformin inhibited *rNIS* after 4 days. In contrast, 1 day of Metformin or Compound C treatment did not result in any changes in *rNIS* activity. This largely corresponds to the observed effects of AMPK modulation on iodide uptake and NIS protein levels, confirming NIS regulation at the transcriptional level. This is in contrast with a study by Andrade et al. (18) where activation of AMPK by AICAR appears to be due to increased degradation/turnover of NIS. Furthermore, in the article by Andrade et al., Compound C was reported to stimulate iodide uptake without increasing NIS expression. A possible explanation was offered suggesting an effect on iodide uptake by a relocation of NIS. These differences may be explained by the use of different cell-lines, compounds or the time of stimulation. Possibly a fast non-transcriptional effect that is present after 24 h and a long-term effect based on increased or decreased transcription of NIS coexist, although in our hands, the effects on iodide uptake were not significant after 24 h.

An important factor in NIS regulation is TSH, which is known as the main regulator of NIS at multiple levels. It positively regulates NIS expression at the protein and mRNA level, increases NIS protein half-life, and facilitates translocation to the plasma membrane (11,12,20,37,38), making a key regulator of iodide uptake. It activates NIS expression by activating the CRE-like element in the NIS promoter (11). We have shown that the CRE element may also be involved in the observed effect of AMPK modulation on NIS gene expression because AMPK modulation had a strong effect on CRE activation. Apparently, the downstream effects of TSH activation and AMPK action seem to synergize on the CRE-like element of the NIS promoter. The fact that the CRE element plays a role in both TSH- and AMPK-mediated signalling may also explain the observation that AMPK inhibition with Compound C increases NIS protein levels even in the absence of TSH. Interestingly, TSH

may also be a key player in AMPK modulation by activating its downstream targets cAMP and protein kinase A (PKA) (39–42), thus adding an additional pathway to TSH stimulated iodide uptake. PKA activation is able to prevent phosphorylation of the catalytic subunit AMPK-alpha at threonine172 and therefore activation of AMPK in several cell lines including follicular thyroid cell lines (18,39–42). Possible mechanisms include the phosphorylation at serine485/491 or serine173 on AMPK, which blocks threonine172 phosphorylation (39,40). Although activation of the CRE element coincides with rNIS promoter activity 4 days following AMPK modulation, CRE activation by compound C at day 1 exceeds rNIS activation. This implies that additional factors are involved, which perhaps is not surprising as CRE-like site activation in the NUE region of the NIS promoter is closely associated with binding of regulatory elements to flanking regions, especially PAX8 (11). Once bound, PAX8 can synergize with a range of b-Zip transcription factors at the CRE-like element site to stimulate NUE activity (36). In contrast to the CRE-like element, activation of the NF-κB element (9) seems to be of minor importance in the activation of NIS following AMPK modulation.

To conclude, we show that NIS expression and iodide uptake can be modulated by metformin and compound C in thyrocytes. It is likely that these compounds exert their effect by modulation of AMPK, which, in turn, regulates the activation of the CRE element in the NIS promoter. The observed effects on iodide uptake and NIS expression suggest that the use of AMPK-modulating compounds may be useful for the enhancement of iodide uptake by thyrocytes, which could be of potential benefit in the treatment of thyroid cancer patients with RAI.

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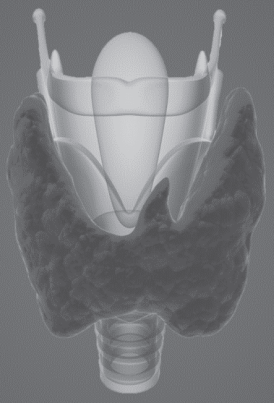
CHAPTER 7

Abnormal cardiac contractility in long-term exogenous subclinical hyperthyroid patients as demonstrated by two-dimensional echocardiography speckle tracking imaging



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ABSTRACT

Background: Subclinical hyperthyroidism is associated with cardiovascular morbidity. Recent advances in echocardiography imaging have allowed sophisticated evaluation of myocardial tissue properties.

Objective: To investigate the myocardial effects of long-term exogenous subclinical hyperthyroidism using two-dimensional speckle tracking echocardiography imaging (2D-STE).

Design: Prospective, single-blinded placebo-controlled randomized trial of 6 months duration with 2 parallel groups.

Patients and methods: Totally 25 patients with a history of differentiated thyroid carcinoma on long-term TSH-suppressive levothyroxine (L-T4) substitution were randomized to persistent TSH-suppressive L-T4 substitution (low-TSH group) or restoration of euthyroidism. Additionally 40 euthyroid controls were studied.

Results: At baseline, the group of patients showed normal left ventricular (LV) systolic function but impaired diastolic function as assessed with conventional echocardiographic parameters. Importantly, 2D-STE analysis demonstrated the presence of subclinical LV systolic and diastolic dysfunction with impaired circumferential and longitudinal strain and strain rate at the isovolumic relaxation time. After restoration of euthyroidism, a significant improvement in LV systolic and diastolic function as assessed with 2D-STE strain was observed.

Conclusion: Prolonged subclinical hyperthyroidism leads to systolic and diastolic dysfunction, which is reversible after restoration of euthyroidism. 2D-STE is a more sensitive technique to evaluate subtle changes in LV performance of these patients.

INTRODUCTION

Subclinical hyperthyroidism is a relatively common thyroid dysfunction with important cardiovascular consequences such as left ventricular (LV) diastolic dysfunction, increased LV mass, and increased risk of supraventricular arrhythmias (1–7). These cardiovascular effects can be accurately evaluated in exogenous subclinical hyperthyroidism, where the duration and development of thyroid dysfunction are well controlled, and different strategies to restore euthyroidism can be performed in a randomized fashion. We recently performed a prospective, randomized; placebo-controlled study in patients treated with total thyroidectomy for differentiated thyroid carcinoma, and demonstrated that 10-year thyroid hormone excess was related to increased LV mass index and overt diastolic dysfunction (7). More importantly, restoration of euthyroidism resulted in normalization of diastolic function.

Recent advances in echocardiography imaging have allowed sophisticated evaluation of myocardial tissue properties that may provide novel insights into the effects of thyroid hormone on the myocardium. Particularly, two-dimensional speckle tracking echocardiography (2D-STE) imaging is a novel technology that permits the study of multidirectional active deformation of the myocardium, providing comprehensive information on myocardial function and closely reflecting myocardial contractile properties (8–10). 2D-STE imaging allows estimation of both strain (radial, longitudinal, and circumferential) and strain rate (SR). Strain measures the myocardial fiber deformation (shortening and thickening), and SR measures the velocity of deformation. The present study explores further the effects of thyroid hormone on the myocardium with the use of 2D-STE and aimed to demonstrate whether LV mechanics, as assessed with multidirectional strain, are impaired in patients with subclinical hyperthyroidism and whether these abnormalities may be reversed after restoration of the euthyroid status. The echocardiographic data collected prospectively in the aforementioned randomized placebo-controlled study were reanalyzed in a blinded fashion with 2D-STE tracking echocardiography and compared with an expanded control group (7).

PATIENTS AND METHODS

The present study was a prospective single-blind randomized study of 6 months duration with two parallel groups. As previously described, the patient population consisted of athyreotic patients subjected to TSH-suppressive thyroxine (T4) treatment (7). Patients had been diagnosed with differentiated thyroid carcinoma and had been initially treated with total thyroidectomy and radioiodide ablative therapy. Cure was documented by the absence of measurable serum thyroglobulin during TSH stimulation and by a negative total-body scintigraphy with 4mCi^{131}I. TSH-suppressive therapy was defined by TSH levels below the lower reference value for normal serum levels of TSH (0.4 mU/l) for at least 10 years.

After inclusion, the patients were randomized in a single-blind fashion to continuation of TSH-suppressive therapy (low-TSH group, with TSH target levels <0.4 mU/l) or restoration of euthyroidism by reducing the levo-T4 (L-T4) dose (euthyroid group, with TSH target levels within the normal reference range 0.40–0.48 mU/l).

Physical examination, hormonal assessments, and comprehensive echocardiographic evaluation were performed at baseline and at 6 months follow-up. Changes in LV hemodynamics and performance were evaluated by conventional echocardiography and by sophisticated 2D-STE analyses. All echocardiographic analyses were performed by a single observer, blinded to treatment modalities.

The echocardiographic findings were compared with a control group of 40 individuals without cardiovascular morbidity: 8 males and 32 females with a mean age 46±8 years. These controls were recruited from an echocardiographic database, as previously described (11). Those individuals with dilated LV, valvular heart disease, or hypertrophic cardiomyopathy were excluded. Accordingly, the control group comprised individuals referred for atypical chest pain, palpitations, or syncope without murmur.

Echocardiography

Patients were imaged in the left lateral decubitus position using a commercially available system equipped with a 3.5 MHz transducer (Vingmed Vivid-7, General Electric Vingmed, Horten, Norway). Standard M-mode, two-dimensional, and color Doppler data were acquired triggered to the QRS complex and saved in cine loop format for off-line analysis (EchoPac 108.1.5, General Electric/Vingmed Ultrasound).

LV dimensions (end-diastolic diameters (EDD) and end-systolic diameters (ESD), end-diastolic interventricular septum thickness, and posterior wall thickness (PWT)), fractional shortening (FS), and LV ejection fraction (LVEF) were measured from M-mode recordings obtained at the parasternal long-axis views, according to the American Society of Echocardiography guidelines (12). LV mass was calculated by Devereux's formula and indexed to body surface area (LVMI) (13).

Diastolic function was evaluated by measuring the following parameters: E-wave, A-wave, E/A ratio, deceleration time (DT) of the E-wave, and isovolumic relaxation time (IVRT) obtained from the pulsed-wave Doppler recordings (14). Finally, left atrial volume was measured from the apical two- and four-chamber views as a morphologic marker of diastolic function.

Two-dimensional speckle tracking strain imaging

2D-STE analysis permits the angle-independent, multidirectional assessment of LV myocardial strain and SR. Both strain and SR characterize the LV tissue mechanical properties and differentiate the active myocardial contraction from the passive motion. Strain is a measure of myocardial deformation and is expressed as percentage (%), whereas SR indicates the rate of the myocardial deformation and is expressed as 1/s. 2D-STE analyses strain and SR by tracking frame-to-frame natural acoustic markers (so-called speckles), equally distributed within the myocardium and visible in the standard grayscale two-dimensional images. Accordingly, LV deformation can be studied along the cardiac cycle in three orthogonal directions as follows: radial, circumferential, and longitudinal (9). Radial and circumferential strains are assessed at the LV mid-ventricular short-axis images. Radial strain measured the thickening/thinning of the myocardial wall, whereas circumferential strain measures the myocardial shortening/lengthening along the curvature of the LV. The mid-ventricular short axis of the LV is divided into six segments, and the global values of radial and circumferential strains are derived from the average of the six segmental peak systolic strain values (Fig. 1, panels A and B). Longitudinal strain is measured at the apical two-chamber, four-chamber, and long-axis views. Longitudinal strain evaluates the shortening/lengthening of the myocardial wall, resulting from the movement of the mitral annulus plane upward/downward the LV apex. Each LV apical view is divided into six segments, and the global longitudinal strain value is derived from the average of the 18

segmental peak systolic strain values (Fig. 1, panel C) (10). Finally, global peak longitudinal SR at the IVRT (SR_{IVRT}) is measured at the three apical views and averaged for final analysis (Fig. 1, panel D), as previously described (15). This parameter is a marker of global myocardial relaxation and reflects the LV filling pressures. Therefore, in the present study, LV mechanical properties were evaluated through three systolic parameters (global radial, circumferential, and longitudinal strains) and one diastolic parameter (SRIVRT), all of them derived with 2D-STE imaging.

Statistical analysis

Continuous variables are presented as mean ± S.D., and categorical variables are presented in number and frequencies. Comparisons between patients and controls were performed with Student's t-test for unpaired data.

As previously described, the effects of different conditions on echocardiographic parameters were evaluated within and between low-TSH group and euthyroid patients using Mann–Whitney U test and Wilcoxon sum rank test for unpaired and paired data respectively. To evaluate the relationship between free T4 and TSH levels and the various echocardiographic parameters, univariate regression analysis was performed. All statistical analyses were performed with SPSS software (version 16.0, SPSS Inc., Chicago, IL, USA). A *P* value <0.05 was considered statistically significant.

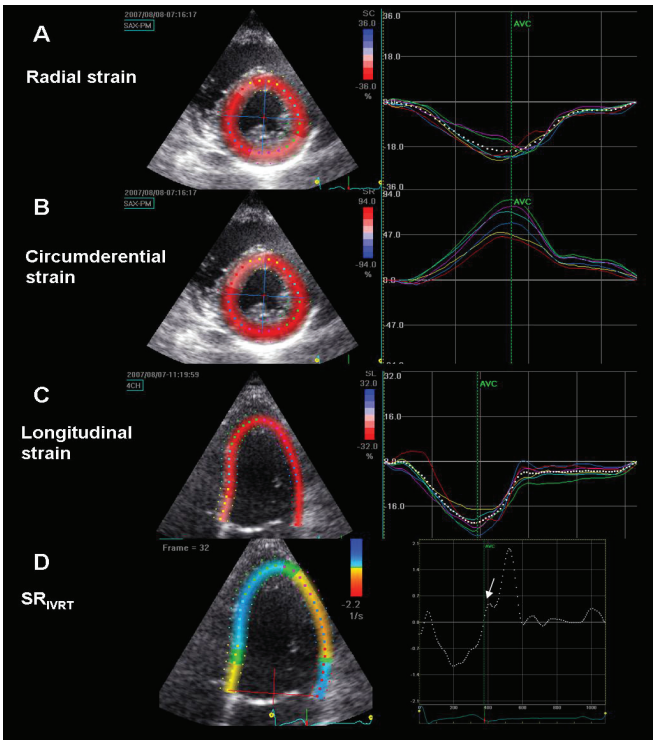

Figure 1 Two-dimensional speckle tracking strain imaging: multidirectional assessment of LV strain and strain rate. At the mid-ventricular short axis view of the LV, radial (panel A) and circumferential (panel B) strain are calculated, obtaining the time-strain curves along the cardiac cycle for the 6 LV segments. Longitudinal strain of the LV is calculated at the apical 4-chamber, 2-chamber and long-axis views (panel C). Global longitudinal strain value is obtained from the average of 18 segments and results are displayed in time-strain curves and polar map. Finally, SRIVRT (panel D) is measured from the LV apical views, as a parameter of LV relaxation. The arrow points out the value of strain rate at the isovolumic relaxation time.

RESULTS

Out of an initial cohort of 33 patients, 25 patients were included in the present study (7). Thirteen patients continued TSH-suppressive therapy (low-TSH group, target TSH level<0.4 mU/l), whereas in the remaining 12 patients, the euthyroid status was restored (euthyroid group). At baseline, weight ((73.2±15.9 (low-TSH group) vs 74.0±9.5 kg (euthyroid group), P=0.881)), systolic blood pressure (135±23 vs 134±14 mm Hg, P=0.920), diastolic blood pressure (81±10 vs 83±5 mm Hg, P=0.728), heart rate (71±7 vs 68±7 bpm, P=0.452), T4 dose (166±34 vs 185±40 µg, P=0.200), TSH (0.054 (<0.005–0.339) vs 0.038 (<0.005–0.302) mU/l, P=0.617), and free T4 (21.7±5.8 vs 22.6±4.0 pmol/l, P=0.637) were not different between the low-TSH group and the euthyroid group. After 6 months, T4 dose (177±33 vs 129±37 µg, P%0.001), TSH concentrations (0.015 (<0.005–0.347) vs 2.66 (0.218–6.090) mU/l, P=0.001) and free T4 (23.1±1.1 vs 18.5±1.1 pmol/l, P<0.001) differed significantly between the low-TSH group and the euthyroid group.

Baseline echocardiography and 2D-STE analysis

At baseline, patients showed significantly higher values of end-diastolic PWT and LV ESD than controls (Table 1). However, LV mass index (LVMI) was comparable between the two groups. In addition, patients had significantly lower values of LV FS and LVEF compared to the group of controls, but within the normal range (Table 1). Regarding the diastolic function, the group of patients showed significantly lower values of E- and A-wave with an inverse E/A ratio and significantly longer values of E-wave DT and IVRT, indicating impaired LV relaxation (Table 1). There were no differences in left atrial volume. Finally, 2D-STE analysis demonstrated a significant impairment of LV circumferential and longitudinal strains in the group of patients, whereas LV radial strain was preserved compared to the group of controls (Table 1). In addition, the SRIVRT was significantly reduced in the group of patients compared to the group of controls, indicating higher LV filling pressures.

Table 1 Baseline echocardiographic characteristics

	Controls (n = 40)	Patients (n = 25)	p-value
LV mass index (g/m^2)	77.2 ± 17.2	86.0 ± 21.0	0.051
IVST (mm)	8.4 ± 1.0	9.2 ± 1.6	0.056
PWT (mm)	8.4 ± 0.9	9.2 ± 1.2	0.003
LV EDD (mm)	49.4 ± 4.7	49.2 ± 4.1	0.989
LV ESD (mm)	28.8 ± 4.5	31.5 ± 4.5	0.020
LV FS(%)	41.4 ± 6.1	36.0 ± 7.3	0.004
LV EF (%)	71.6 ± 7.1	64.7 ± 9.3	0.002
E-wave (cm/s)	72.9 ± 17.6	55.3 ± 9.5	<0.001
A-wave (cm/s)	56.7 ± 12.6	63.9 ± 10.8	0.029
E/A ratio	1.3 ± 0.2	0.87 ± 0.13	<0.001
E-wave DT (ms)	191.1 ± 33.3	234 ± 34	<0.001
IVRT (ms)	77.7 ± 13.3	121.0 ± 15.0	<0.001
Left atrial volume (ml)	45.5 ± 13.7	50.5 ± 13.7	0.160
E'-wave (cm/s)	9.2 ± 1.7	5.7 ± 1.3	<0.001
A'-wave (cm/s)	6.5 ± 1.4	6.8 ± 1.4	0.494
E'/A' ratio	1.4 ± 0.5	0.89 ± 0.35	<0.001
Radial strain (%)	43.9 ± 17.5	42.3 ± 11.8	0.687
Circumferential strain (%)	-19.7 ± 2.8	-16.9 ± 2.3	0.001
Longitudinal strain (%)	-19.9 ± 2.8	-17.7 ± 1.2	<0.001
SR_{IVRT} (1/s)	0.39 ± 0.09	0.29 ± 0.08	<0.001

Abbreviations: DT = deceleration time; EDD = end-diastolic diameter; EF = ejection fraction; ESD = end-systolic diameter; FS = fractional shortening; IVRT = isovolumic relaxation time; IVST = interventricular septum thickness; LV = left ventricular; PWT = posterior wall thickness; SR_{IVRT} = strain rate at the isovolumic relaxation time.

Low-TSH group versus euthyroid group: conventional echocardiography

At 6 months follow-up, the euthyroid group showed significant reductions in LV dimensions and improvements in LV FS and LVEF (Table 2). In addition, LV diastolic function improved significantly in the euthyroid group as indicated by a reduction in the A-wave velocity and normalization of the E/A ratio, a significant reduction in the left atrial volume, and significant decreases in E-wave DT and IVRT. In contrast, the low-TSH group exhibited a significant increase in LV dimensions (EDDs and ESDs) and a significant reduction in LV FS and LVEF (Table 2). No changes in conventional imaging-derived LV diastolic parameters were observed in the low-TSH group.

Table 2 Low-TSH group vs. euthyroid group: conventional echocardiography and tissue Doppler imaging

	Low-TSH (n= 13)		Euthyroid (n = 12)		
	Baseline	6-months	Baseline	6-months	p-value$^{\alpha}$
LV mass index (g/m^2)	86.0 ± 26.0	89.0 ± 12.0	87.0 ± 16.0	95.0 ± 24.0	0.468
IVST (mm)	8.9 ± 1.9	9.2 ± 1.1	9.4 ± 1.4	10.0 ± 1.6	0.630
PWT (mm)	9.0 ± 1.2	8.9 ± 1.2	9.5 ± 1.3	9.6 ± 0.9	0.763
LV EDD (mm)	49.1 ± 4.0	52.1 ± 3.3	49.3 ± 4.5	48.6 ± 3.3	0.045
LV ESD (mm)	30.1 ± 4.0	34.1 ± 5.4*	33.1 ± 4.8	29.5 ± 2.5*	<0.001
LV FS (%)	38.4 ± 7.0	34.8 ± 8.0*	33.3 ± 7.2	39.4 ± 3.8*	<0.001
LV EF (%)	67.6 ± 8.6	62.6 ± 11.4*	61.3 ± 9.6	69.8 ± 4.6*	<0.001
E-wave (cm/s)	58.1 ± 7.9	58.9 ± 8.2	51.0 ± 10.0	54.8 ± 13.3	0.353
A-wave (cm/s)	66.1 ± 11.2	67.7 ± 12.4	61.3 ± 10.6	47.4 ± 11.6*	<0.001
E/A ratio	0.91 ± 0.16	0.88 ± 0.12	0.83 ± 0.10	1.18 ± 0.27*	<0.001
E-wave DT (ms)	230 ± 34	236 ± 26	237 ± 38	196 ± 24*	0.001
IVRT (ms)	115 ± 15	121 ± 17	127 ± 13	95 ± 15*	<0.001
Left atrial volume (ml)	48.6 ± 14.7	50.7 ± 17.4	52.0 ± 12.4	44.9 ± 12.4*	0.009
E'-wave (cm/s)	5.9 ± 1.8	5.2 ± 1.8	5.5 ± 0.6	7.2 ± 1.3*	<0.001
A'-wave (cm/s)	6.4 ± 1.4	5.7 ± 1.5	7.1 ± 1.4	6.7 ± 1.6	0.493
E'/A' ratio	0.97 ± 0.42	0.97 ± 0.34	0.81 ± 0.26	1.13 ± 0.34*	0.003

Abbreviations: DT = deceleration time; EDD = end-diastolic diameter; EF = ejection fraction; ESD = end-systolic diameter; FS = fractional shortening; IVRT = isovolumic relaxation time; IVST = interventricular septum thickness; LV = left ventricular; PWT = posterior wall thickness.
*p <0.05 vs. Baseline (wihtin the groups); $^{\alpha}$Difference between 6 months and baseline, euthyroid *vs.* low-TSH group

Low-TSH group versus euthyroid group: 2D-STE analysis

At baseline, both groups of patients showed comparable values of LV radial, circumferential, and longitudinal strains and comparable values of SR_{IVRT} as measured with 2D-STE imaging (Table 3). At 6 months follow-up, the euthyroid group showed a significant improvement in

LV circumferential and longitudinal strains and in SR_{IVRT}, whereas no changes in LV radial strain were observed (Fig. 2). In contrast, the low-TSH group did not have significant changes in multidirectional LV strain or SR_{IVRT} (Fig. 2). There were significant differences in changes in circumferential and longitudinal strains and in SR_{IVRT} between the two groups (Table 3).

Table 3 Low-TSH group vs. euthyroid group: 2-dimensional speckle tracking strain imaging

	Low-TSH (n= 13)		Euthyroid (n = 12)		
	Baseline	6-months	Baseline	6-months	p-valueα
Radial strain (%)	42.9 ± 10.2	39.7 ± 13.7	41.7 ± 13.7	42.0 ± 11.2	0.431
Circumferential strain (%)	-17.0 ± 2.8	-16.5 ± 1.3	-16.9 ± 1.7	-18.8 ± 2.3*	0.018
Longitudinal strain (%)	-17.9 ± 1.3	-17.5 ± 1.8	-17.5 ± 1.1	-19.7 ± 2.1*	0.001
SRIVRT (1/s)	0.29 ± 0.1	0.28 ± 0.08	0.29 ± 0.06	0.42 ± 0.09*	0.004

Abbreviations: SR_{IVRT} = strain rate at the isovolumic relaxation time.
*p <0.05 vs. Baseline (wihtin the groups); αDifference between 6 months and baseline, euthyroid vs. low-TSH group

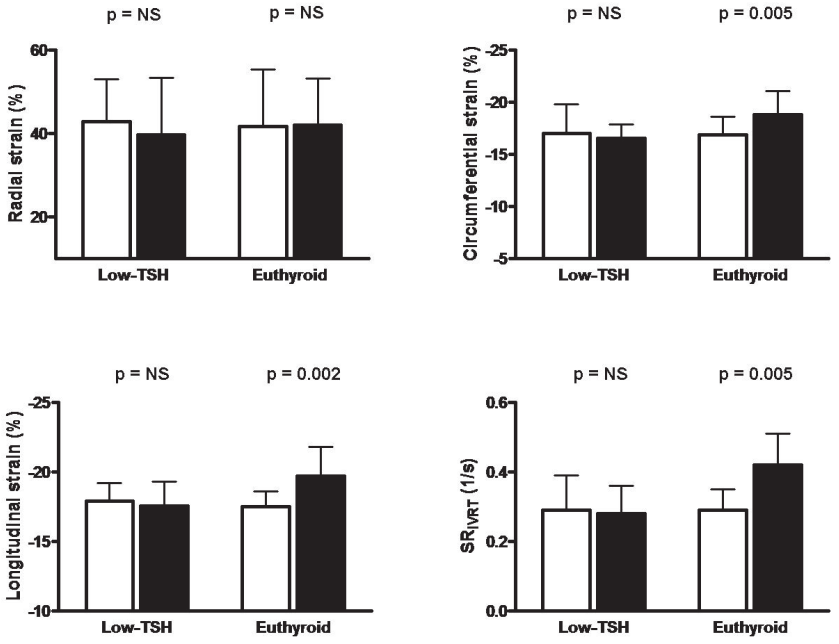

Figure 2 Changes in multidirectional LV strain and SR_{IVRT} in low- TSH and euthyroid groups. The bar graphs show multidirectional LV strain and strain rate values at baseline (white bars) and follow-up (solid bars) in the low-TSH group and the euthyroid group. In those patients who continued TSH-suppressive therapy, there were no changes in multidirectional LV strain and strain rate at the isovolumic relaxation time (SR_{IVRT}). In contrast, those patients with restoration of the euthyroid status showed significant improvements in circumferential and longitudinal strains and in SR_{IVRT}

Relationship between TSH levels and echocardiographic parameters at follow-up

Univariate regression analysis was used to evaluate the influence of free T4 and TSH levels on different echocardiographic parameters (Table 4). Free T4 and TSH levels were not related to LV dimensions or systolic function as measured with conventional parameters. In contrast, some LV diastolic parameters as measured with transmitral pulsed-wave Doppler echocardiography (A-wave, E/A ratio, DT, and IVRT) or tissue Doppler imaging (E'-wave) were significantly related to free T4 and TSH levels. Finally, novel indices of LV systolic function based on 2D-STE analysis showed a borderline association with TSH levels but not with free T4 levels.

Table 4 Univariate regression analysis between free thyroxine (T4) and TSH levels and echocardiographic parameters at follow-up.

	T4 (pmol/l)			TSH (mU/l)		
	β	95% confidence interval	*P* value	β	95% confidence interval	*P* value
LV mass index (g/m^2)	0.286	- 0.770 to 3.294	0.209	0.176	- 1.746 to 4.113	0.411
IVST (mm)	0.148	- 0.100 to 0.190	0.521	0.174	- 0.166 to 0.385	0.827
PWT (mm)	0.067	- 0.095 to 0.126	0.774	0.232	- 0.097 to 0.325	0.275
LV EDD (mm)	0.126	- 0.295 to 0.508	0.586	0.138	- 0.813 to 0.424	0.521
LV ESD (mm)	0.120	- 0.405 to 0.676	0.606	0.335	- 1.486 to 0.161	0.109
LV FS (%)	0.089	- 0.870 to 0.597	0.701	0.324	- 0.278 to 2.188	0.123
LV EF (%)	0.094	- 1.250 to 0.839	0.685	0.331	- 0.362 to 3.132	0.114
E-wave (cm/s)	0.037	- 1.243 to 1.067	0.875	0.103	- 2.741 to 1.697	0.630
A-wave (cm/s)	0.438	- 2.924 to - 0.020	0.047	0.672	- 12.672 to - 4.376	<0.001
E/A ratio	0.413	- 0.001 to 0.043	0.063	0.587	- 6.911 to -1.677	0.003
E-wave DT (ms)	0.112	- 2.692 to 4.351	0.628	0.575	0.025 to 0.110	0.003
IVRT (ms)	0.153	- 2.593 to 1.327	0.507	0.459	- 7.747 to - 0.597	0.024
Left atrial volume (ml)	0.407	- 2.316 to 0.088	0.067	0.030	- 3.285 to 2.871	0.890
E'-wave (cm/s)	0.461	0.012 to 0.308	0.036	0.446	0.040 to 0.665	0.029
A'-wave (cm/s)	0.191	- 0.102 to 0.241	0.407	0.230	- 0.147 to 0.484	0.289
E'/A' ratio	0.231	- 0.012 to 0.036	0.313	0.213	- 0.033 to 0.097	0.319
Radial strain (%)	0.152	- 1.061 to 1.970	0.535	0.386	- 0.202 to 4.937	0.069
Circumferential strain (%)	0.044	- 0.246 to 0.207	0.857	0.389	- 0.837 to 0.030	0.067
Longitudinal strain (%)	0.243	- 0.270 to 0.085	0.288	0.377	- 0.760 to 0.031	0.069
SR_{IVRT} (1/s)	0.035	- 0.011 to 0.012	0.879	0.298	- 0.006 to 0.035	0.157

DT, deceleration time; EDD, end-diastolic diameter; EF, ejection fraction; ESD, end-systolic diameter; FS, fractional shortening; IVRT, isovolumic relaxation time; IVST, interventricular septum thickness; LV, left ventricular; PWT, posterior wall thickness.

DISCUSSION

The present study provides new insights into the effects of thyroid hormone on myocardial mechanical performance assessed with 2D-STE. In patients with long-term exogenous

subclinical hyperthyroidism and preserved LVEF, 2D-STE analysis demonstrated the presence of impaired LV myocardial deformation. After restoration of euthyroid status, a significant improvement in circumferential and longitudinal strains and SRIVRT was observed. Therefore, in patients with exogenous subclinical hyperthyroidism and preserved LVEF, the excess of thyroid hormone exerts a deleterious effect on myocardial function that is reversible upon restoration of euthyroid status. It remains to be determined whether these changes may be reversed or not once overt heart failure is present.

The cardiovascular effects of subclinical hyperthyroidism have been well documented, and include increased LV mass, diastolic dysfunction, and increased risk of cardiac arrhythmias (1–6, 16). However, it is important to note that previous studies included retrospective series and yielded observational and controversial results (4, 16–18). In contrast, the randomized design of the present study permitted evaluation of the direct cardiovascular effects of thyroid hormone. Patients with subclinical hyperthyroidism showed impaired diastolic function as assessed with conventional pulsed-wave Doppler echocardiography and tissue Doppler imaging (7). After restoration of euthyroid hormone levels, significant improvements in diastolic function were observed. The present study represents a step further in the characterization of LV diastolic performance by evaluating the relaxation properties of the myocardium with 2D-STE. At baseline, the overall population showed a reduced SR_{IVRT}, a surrogate of increased LV filling pressures (15). Improvement in LV filling pattern with an increase in SRIVRT was only observed in those patients with restored euthyroidism, whereas in those patients with low-TSH levels, SR_{IVRT} remained unchanged. The measurement of SR_{IVRT} provides additional insight to previous findings based on tissue Doppler imaging, as this parameter may be less load-dependent and relies on active deformation of the myocardium. In addition, these changes in LV diastolic dysfunction were independent of LV mass. The present study population had an increased LV mass as compared with controls. However, most patients did not show LV hypertrophy. As previously demonstrated, thyroid hormone exerts direct effects on myocardial diastolic relaxation independent of protein synthesis and cardiac growth (1, 19). Therefore, it may reflect more the direct biochemical effects of thyroid hormone on the myocardium, which lead to activation of local signal transduction pathways rather than the effects of LV hypertrophy (1, 20). In earlier studies, more profound LV hypertrophy was found that was reversed by b-blockers (5, 21, 22) or dose reduction (23). These studies also included patients with multinodular goiter, who may have

been exposed to higher levels of free T4 and have subsequently shown more profound myocardial hypertrophy.

It can be hypothesized that the fact that we did not find a significant difference between patients and controls in LVMI and IVST could be explained by the fact that TSH levels were not suppressed in all patients. We therefore compared IVST and LVMI between patients with completely suppressed TSH levels at baseline and patients with TSH R0.1 mU/l, but did not find significant differences between these two groups.

The most insightful finding of the present study is, perhaps, the presence of subclinical LV systolic dysfunction as assessed by means of multidirectional strain. In patients with subclinical hyperthyroidism, the prevalence of overt LV systolic dysfunction as assessed with conventional two-dimensional echocardiography (LVEF <45%) is rather low (2.4%) (24). However, it has been shown that despite preserved or even increased LVEF at rest, patients with thyroid hormone excess may have impaired LV contractile reserve with no further increase in LVEF during exercise (25). Myocardial strain analysis based on 2D-STE may be a more sensitive tool than conventional measurements of systolic LV function, as it provides information on active deformation of the myocardium. In addition, this imaging tool enables the evaluation of subtle changes in myocardial strain after restoration of euthyroidism, as demonstrated in the present study.

These findings may have important clinical implications as long-term subclinical hyperthyroidism has been related to increased incidence of cardiovascular events (6). The tight control of thyroid hormone level with restoration of euthyroidism may help to improve the cardiovascular risk profile of these patients and therefore may reduce the risk of cardiovascular events. In this regard, 2D-STE may allow refining the therapy of this group of patients.

Some limitations have to be acknowledged. First, the assessment of LV myocardial function with 2D-STE analysis after an acute change of TSH or free T4 was not performed, and therefore the effects of these hormones at the tissue level could not be elucidated. In addition, the small study population may preclude us from observing stronger relationships between the 2D-STEderived parameters of LV function and TSH levels.

In conclusion, patients with prolonged subclinical hyperthyroidism show subtle LV systolic and diastolic dysfunction, which is reversible after restoration of euthyroidism.

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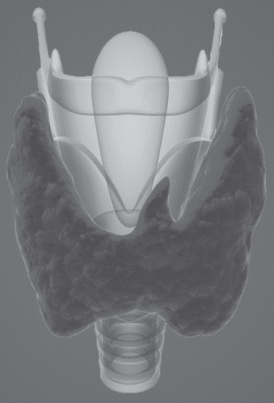
CHAPTER 8

Both exogenous subclinical hyperthyroidism and short-term overt hypothyroidism affect myocardial strain in patients with differentiated thyroid carcinoma



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ABSTRACT

Background: The cardiovascular effects of transitions from exogenous subclinical hyperthyroidism to short-term overt hypothyroidism in patients treated for differentiated thyroid carcinoma (DTC) remain unclear. The present study aims to evaluate the changes in multidirectional myocardial strain using 2-dimensional (2D) speckle tracking during this controlled transition from exogenous subclinical hyperthyroidism to overt hypothyroidism.

Patients and methods: The study included 14 patients with DTC on TSH suppressive thyroxine substitution who were subsequently withdrawn from thyroxine for 4 weeks. Cardiac function was assessed by 2D speckle tracking echocardiography before, and 1 and 4 weeks after withdrawal and compared to values obtained in a control group of 24 individuals.

Results: At baseline the LV dimensions were significantly higher in patients as compared to controls. Using 2D speckle tracking imaging, the patients had significantly impaired baseline myocardial systolic function in the circumferential (-16.0±2.1% vs. -19.2±3.0%, p=0.001) and longitudinal (-17.1±2.5% vs. -19.7±3.0%, p=0.001) directions as compared to controls. Withdrawal of thyroid hormone did not induce significant changes in LV dimensions or systolic function. During the transition from exogenous subclinical hyperthyroidism to overt hypothyroidism, a significant improvement in circumferential and longitudinal systolic shortening was observed, and returned to abnormal values when the patients were overt hypothyroid (circumferential strain: from -16.0±2.1% to -18.6±1.9% and -14.7±2.8%, p<0.005; longitudinal strain: from -17.1±2.5% to -18.8±1.4% and -16.3±1.3%, p<0.005).

Conclusions: A U-shaped relationship between a range of thyroid hormone levels (from hyper- to hypothyroid concentrations) and myocardial strain was observed. The clinical consequences of these findings remain to be determined, but may point out an increased myocardial vulnerability even in states of moderate subclinical hyperthyroidism and short-term hypothyroidism.

INTRODUCTION

The cardiovascular consequences of hyper- and hypothyroidism have been extensively described and include systolic and diastolic left ventricular (LV) dysfunction, increased LV mass and cardiac arrhythmias (1-6). Many of these studies have been performed in patients with endogenous thyroid disorders and used cross-sectional designs. Ideally, effects of thyroid hormone on myocardial function should be studied in subjects being exposed in a controlled fashion to a wide spectrum of plasma thyroid hormone levels. Patients with differentiated thyroid carcinoma (DTC), who until recently were substituted with TSH suppressing dosages of levothyroxine and routinely withdrawn from thyroid hormone substitution for diagnostic studies, offer an attractive group to perform this type of studies as they undergo short term controlled transitions from subclinical hyperthyroidism to hypothyroidism. Results from earlier published studies, including our own, on myocardial effects of short-term thyroid hormone withdrawal however have been inconclusive, varying from mainly decreased diastolic function to mainly altered systolic function (7-15). These studies have all been performed with conventional echocardiographic parameters, which may not be sensitive enough to detect subtle abnormalities at the myocardial level.

Currently, 2-dimensional (2D) speckle tracking echocardiography permits the multidirectional evaluation of the mechanical properties of the myocardial tissue (16-18). Importantly, active myocardial deformation can be evaluated providing information on myocardial contractility. Therefore, this novel technique may permit the detection of subtle effects of thyroid hormone at the myocardial level. Accordingly, we re-analyzed data from the aforementioned study (13), to comprehensively evaluate LV performance in relation to a wide spectrum of plasma thyroid hormone levels in DTC patients in transition from subclinical hyperthyroidism, through euthyroidism to hypothyroidism and to detect changes in LV function using 2D speckle tracking echocardiography.

PATIENTS AND METHODS

Patient population and study design

The patient population consisted of 14 patients in whom DTC was diagnosed and who were subsequently treated with total thyroidectomy and radioiodide ablative therapy. The patient characteristics and the study design have been previously reported (13).In brief, after ablative

therapy, the patients received TSH-suppressive thyroxine replacement therapy, aiming at TSH levels <0.1 mU/L (normal reference values 0.4-4.4 mU/L). The patients were scheduled for TSH stimulated diagnostic studies (including thyroglobulin measurements and iodide-131 whole body scanning to evaluate the effect of prior radioiodide therapy). Patients were routinely withdrawn from thyroxine therapy during approximately 28 days. On the last day of thyroxine therapy (visit 1) and at days 7 (visit 2) and 28 (visit 3) after thyroid hormone withdrawal, biochemical and hormonal parameters were evaluated and 2D transthoracic echocardiography was performed. The changes in biochemical, hormonal and conventional echocardiographic parameters have been previously reported (13). Additionally, LV strain was evaluated with 2D speckle tracking imaging in order to characterize the mechanical properties of the myocardial tissue. As previously described, none of the patients had previous myocardial infarction, rheumatic fever, endocarditis, diabetes mellitus or connective tissue disorders (13). In addition, the patients were hemodynamically stable during the study period and any treatment that could influence cardiovascular parameters was not used.

The study was approved by the local ethics committee and written informed consent was obtained from all patients.

Finally, 24 individuals matched by age, gender, body surface area and LV ejection fraction (LVEF) were included in the present study and formed the control group. All controls were clinically referred to evaluate atypical complaints and rule out structural heart disease. Individuals referred for echocardiographic evaluation of known valvular heart disease, murmur, congestive heart failure, and cardiac transplantation were excluded. In addition, those individuals with any cardiovascular co-morbidity were excluded. Therefore, the controls showed normal echocardiographic studies and provided the normal reference values for the evaluated echocardiographic parameters (19).

Echocardiography

Echocardiography was performed with the patients in the left lateral decubitus position and using a commercially available system equipped with a 3.5-MHz transducer (Vingmed Vivid-7, General Electric Vingmed, Horten, Norway). Standard M-mode, 2-D and color-Doppler data were acquired, triggered to the QRS complex, and saved in cine-loop format for off-line analysis (EchoPac 108.1.5, General Electric/Vingmed Ultrasound). From the M-mode recordings obtained at the parasternal long-axis views, LV dimensions (end-diastolic and end-

systolic diameters, end-diastolic interventricular septum thickness and posterior wall thickness), fractional shortening and LVEF were measured, according to current guidelines (19). LV mass was calculated by Devereux's formula and indexed to body surface area (20).

2D speckle tracking echocardiography analysis

2D speckle tracking echocardiography permits the angle-independent, multidirectional assessment of LV myocardial strain and strain rate (16). As previously described, the left ventricle deforms in three orthogonal directions (radial, circumferential and longitudinal) (16). Strain imaging characterizes the LV tissue mechanical properties and differentiates the active myocardial contraction from the passive motion. Strain is a measure of myocardial deformation and is expressed as percentage (%).

From the LV mid-ventricular short-axis images, radial and circumferential strains are assessed. Radial strain measures the thickening/thinning of the myocardial wall, whereas circumferential strain measures the myocardial shortening/lengthening along the curvature of the LV. The average of the six segmental peak systolic strain values provides the global values of radial and circumferential strain (Fig 1A, B chapter 7, p. 97).

Longitudinal strain is measured at the apical 2-, 4-chamber and long-axis views. Longitudinal strain evaluates the shortening/lengthening of the myocardial wall, resulting from the movement of the mitral annulus plane upward/downward the LV apex. The average of the 18 segmental peak systolic strain values provides the global longitudinal strain value (Fig 1C chapter 7, p. 97) (17).

Therefore, in the present study, LV mechanical properties were evaluated through three systolic parameters (global radial, circumferential and longitudinal strains), all of them derived with 2D speckle tracking strain imaging.

Statistical analysis

Continuous data are presented as mean ± standard deviation whereas categorical data are presented as number and frequencies. Baseline echocardiographic characteristics were compared between the patients and the controls using Kruskal-Wallis test. A p-value <0.05 was considered statistically significant. Changes in clinical and echocardiographic parameters within the 3 different time points (baseline [visit 1], 7 days [visit 2] and 28 days [visit 3]) were compared with the Friedman test and to adjust for inflation of the type I error with multiple

tests, a posthoc correction was applied; consequently, a p-value < 0.017 was considered significant (0.05 divided by 3 different stages). All statistical analyses were performed with SPSS software (version 16.0, SPSS Inc., Chicago, Illinois).

RESULTS

Patient characteristics

This study included 14 patients with DTC (3 men and 11 women), age 51.6 ± 14.5 years, without distant metastases. Median time of TSH suppressive levothyroxine therapy was 1 year. L-thyroxine dose before the withdrawal was 162.5± 41.6 μg/day. As previously described, at visit 1, serum free thyroxine concentrations were above the upper limit of the normal range (reference range 10-24 pmol/l), TSH (reference range 0.4-4.8 mU/l) levels were below normal range and thyronine (reference range 1.1-3.6 nmol/l) within normal range (13). After 7 days of thyroxine withdrawal, free thyroxine levels were already slightly below the lower limit of the reference values, and TSH levels had increased significantly, whereas thyronine levels were still within normal range. During the transition period from hyper- to hypothyroidism the patients showed a significant increase in body mass index and the diastolic blood pressure (Table 1).

The control group included 24 individuals (45.4±8.5 years, 3 men). By design, there were no differences between controls and patients for age, gender, and body surface area. In addition, there were no significant differences in systolic and diastolic blood pressure and heart rate between healthy controls and patients (Table 1).

LV dimensions and function as assessed with conventional echocardiographic parameters

As previously reported, at baseline the LV dimensions were significantly higher in patients in comparison to controls (Table 1). However, none of the patients met the criteria for LV hypertrophy (13). As previously reported, the acute withdrawal of thyroid hormone (visit 2) did not induce significant changes in LV dimensions or systolic function (Table 1). In addition, these conventional echocardiographic parameters remained unchanged after 28 days (visit 3) of thyroid hormone withdrawal (Table 1).

Changes in LV myocardial tissue mechanical properties as assessed with 2D speckle tracking echocardiography

At baseline, the evaluation of the myocardial tissue properties with 2D speckle tracking imaging demonstrated that the group of patients had significantly impaired myocardial systolic function in the circumferential and longitudinal directions as compared to the group of controls (Table 1). In contrast, radial strain was preserved.

Remarkably, after short-term withdrawal of thyroid hormone, when the patients had the hormone levels within the euthyroid range (visit 2), a significant improvement in circumferential and longitudinal systolic shortening was observed (Fig 2). However, after 28 days of thyroid hormone withdrawal, when the patients were in an overt hypothyroid state (visit 3), circumferential and longitudinal strains significantly impaired (Fig 2). Radial strain values remained unchanged during the study as it reflects the mid-wall myocardial layer which is only affected at late stages of cardiac dysfunction. Therefore, 2D speckle tracking permitted the detection of subtle changes in myocardial tissue mechanical properties during the acute induction of hypothyroidism.

DISCUSSION

The current study evaluated subtle changes in LV function using 2D speckle tracking echocardiography in relation to a wide spectrum of plasma thyroid hormone levels in DTC patients in transition from exogenous subclinical hyperthyroidism via euthyroidism to overt hypothyroidism. Exogenous subclinical hyperthyroid status was associated with impaired myocardial deformation in the circumferential and longitudinal directions. Restoration of euthyroidism induced an improvement in myocardial deformation in these two directions whereas overt hypothyroidism was accompanied by impairment in myocardial deformation properties. In contrast, radial strain was preserved along the follow-up. As previously described (21), the LV myocardial architecture is complex array of longitudinally and circumferentially orientated fibers located at the subepi/subendocardium and mid-wall, respectively. The subendocardial fibers are more vulnerable to ischemia, hemodynamic overload or age-related changes and therefore longitudinal strain is commonly impaired at an early stage of the disease. In contrast, radial strain, derived from mid-wall fibers, is preserved.

Table 1. Basic Clinical , Biochemical And Echocardiographic Findings Of The Patients And Controls

	Controls	Visit 1 (subclinical hyperthyroidism)	Visit 2 (7 days after withdrawal)	Visit 3 (28 days after withdrawal)
Clinical parameters				
Men/Women (number)	3/21	3/11	3/11	3/11
Age (years)	45.4±8.5	51.6 ± 14.5	51.6 ± 14.5	51.6 ± 14.5
Body mass index (kg/ m2)	24.9 ± 3.1	26.5 ± 6.1	26.9 ± 5.9[a]	27.6 ± 6.0[a,b]
Systolic blood pressure (mmHg)	124.8 ± 7.7	130.1 ± 23.2	130.2 ± 23.3	131.3 ± 20.1
Diastolic blood pressure (mmHg)	76.0 ± 6.9	81.7 ± 16.5	77.6 ± 12.9	85.5 ± 10.4[b,c]
Heart rate (beats per minute)	70.4 ± 8.4	70.8 ± 8.0	65.2 ± 8.2[a]	66.6 ± 6.7
Assays				
fT4 (pmol/ L) (ref. 10–24 pmol/ L)	NA	26.4 ± 3.1	9.6 ± 1.9[a]	2.2 ± 1.3[a,d]
TSH (mU/ L) (ref.0·4–4·8 mU/ L)	NA	0.3 ± 0.58	9.5 ± 15.5[a]	105.2 ± 57.8[a,b]
T4 (nmol/L) (ref. 1.1-3.6 nmol/L)	NA	1.5 ± 0.44	1.1 ± 0.19[a]	0.6 ± 0.23[a,b]
2D echocardiography				
LV mass index (g/m^2)	73.6 ± 9.3	81.6 ± 12.1[c]	84.1 ± 12.2[c]	85.2 ± 16.7[c]
IVST (mm)	8.3 ± 1.0	9.9 ± 1.3	9.3 ± 1.4	9.5 ± 1.5
PWT (mm)	8.2 ± 0.7	9.5 ± 1.9	9.4 ± 1.2	9.7 ± 1.5
LVEDD (mm)	48.5 ± 4.5	46.9 ± 5.3	49.4 ± 5.0	48.9 ± 5.0
LVESD (mm)	28.1 ± 4.1	28.7 ± 4.6	29.6 ± 3.5	29.1 ± 3.2
LVFS (%)	38.2 ± 3.4	38.4 ± 7.4	39.8 ± 5.7	40.1 ± 5.8
LVEF (%)	68.0 ± 4.2	68.0 ± 8.8	70.4 ± 6.1	70.3 ± 6.7
2D speckle tracking				
Radial strain (%)	43.1 ± 18.5	36.5 ± 16.0	39.8 ± 15.0	36.6 ± 12.4
Circumferential strain (%)	-19.2 ± 3.0	-16.0 ± 2.1[c]	-18.6 ± 1.9[a]	-14.7 ± 2.8[b,c]
Longitudinal strain (%)	-19.7 ± 3.0	-17.1 ± 2.5[c]	-18.8 ± 1.4[a]	-16.3 ± 1.3[b,c]

[a]P<0.017 compared with visit 1.
[b]P<0.017 compared with visit 2.
[c]P≤0.05 compared with controls.
2D, two-dimensional; IVST, interventricular septum thickness; LV, left ventricular; LVEDD, left ventricular end-diastolic diameter; LVEF, left ventricular ejection fraction; LVESD, left ventricular end-systolic diameter; LVFS, left ventricular fractional shortening; PWT, posterior wall thickness; ref, reference ranges used in the Leiden University Medical Center.

Patients with DTC, who until recently were substituted with TSH suppressing dosages of levothyroxine and routinely withdrawn from thyroid hormone substitution for diagnostic studies, offer an attractive group to perform this type of studies. Particularly, the design of the present study permitted to evaluate subtle changes in LV myocardial performance providing a reliable and sensitive evaluation of the effects of thyroid hormone at the myocardial level.

Earlier studies have been performed using conventional echocardiographic strategies, which may not be sensitive enough to detect subtle abnormalities at the myocardial level.

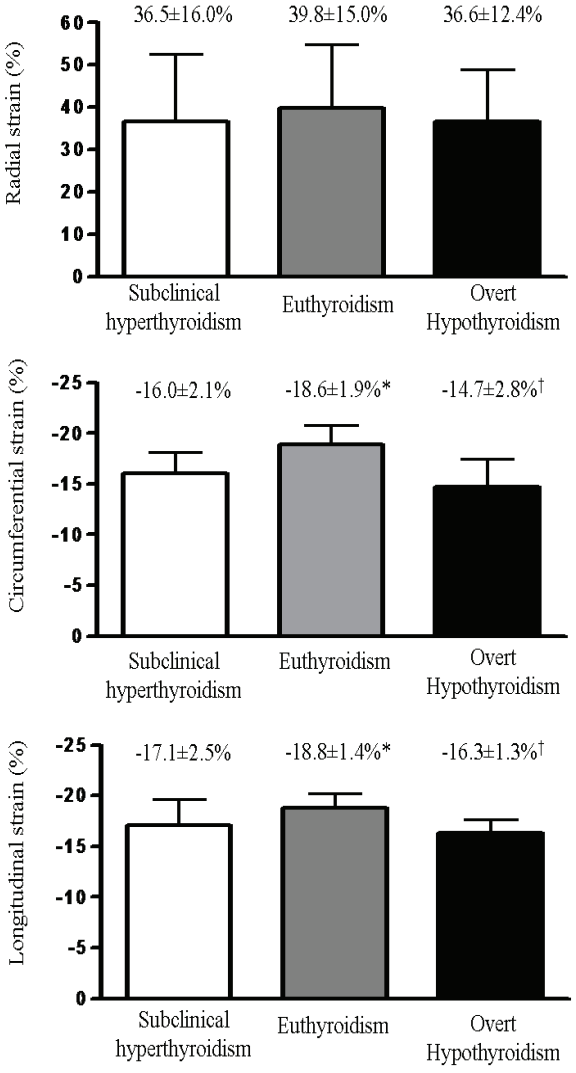

Fig 2: Changes in multidirectional LV myocardial functions during thyroid hormone withdrawal. Baseline values of LV multidirectional strain are presented for patients at baseline (visit 1), 7 (visit 2) and 28 (visit 3) days after thyroid hormone withdrawal. After 7 days of thyroid hormone withdrawal (visit 2, euthyroid to hypothyroid status), a significant improvement was observed in circumferential and longitudinal strain. However, after 28 days of thyroid hormone withdrawal (visit 3, overt hypothyroidism), circumferential and longitudinal strain significantly impaired. Radial strain remained unchanged during the follow-up.
* $p<0.017$ vs. visit 1; †$p<0.017$ vs. visit 2

Indeed, in those studies, no clear conclusions on the cardiac consequences of acute hypothyroidism in patients with exogenous subclinical hyperthyroidism were reached (7-15). None of those studies focused on the direct effect of thyroid hormone on myocardial contractility and deformation. 2D speckle tracking strain imaging enables assessment of systolic and diastolic myocardial deformation properties in the 3 orthogonal spatial directions.

Unlike tissue Doppler imaging, 2D speckle tracking imaging provides angle-independent measurements of myocardial strain. Although conventional echocardiography and tissue Doppler imaging did not show any significant changes, 2D speckle tracking strain imaging showed remarkable results, with significantly decreased circumferential and longitudinal strain as compared with controls during subclinical hyperthyroidism, a significant improvement during euthyroidism, and a significant deterioration again during profound hypothyroidism. Therefore, a remarkable U-shaped relationship between thyroid hormone concentrations and myocardial strain was observed, with hyper- and hypothyroidism equally affecting myocardial mechanical properties. As such, this study demonstrates the myocardial effects of a wide spectrum of thyroid hormone levels independently of LV mass. Indeed, it has been previously demonstrated, that thyroid hormone exerts direct effects on myocardial diastolic relaxation independent of protein synthesis and cardiac growth (5, 22). Therefore, the changes observed in myocardial strain may reflect the direct biochemical effects of thyroid hormone on the myocardium that lead to activation of local signal transduction pathways rather than changes in LV hypertrophy (23). In addition, changes in hemodynamic conditions that take place during transition from exogenous subclinical hyperthyroidism to euthyroidism and overt hypothyroidism may also influence myocardial strain (24). Whether these changes in multidirectional myocardial strain may influence the TSH suppressive treatment and, subsequently, the long-term outcome remain unknown. Additional studies are needed to elucidate the effects on long-term outcome of a tailored TSH suppressing therapy based on 2D speckle tracking strain imaging.

Some study limitations should be acknowledged. Circulating levels of TSH and free thyroxine were not systematically determined in all controls. Therefore, the controls were considered euthyroid based on clinical history and complete physical examination.

In conclusion this study demonstrates the subtle effects of profound short term changes of thyroid hormone levels on the myocardium, likely reflecting direct biochemical effects of thyroid hormone on myocardial metabolism, rather than structural changes. A U-shaped relationship between the spectrum of thyroid hormone levels from hyper- to hypothyroid concentrations and myocardial strain is observed. The clinical consequences of these findings remain to be determined, but may point to an increased myocardial vulnerability even in states of moderate subclinical hyperthyroidism and short term hypothyroidism.

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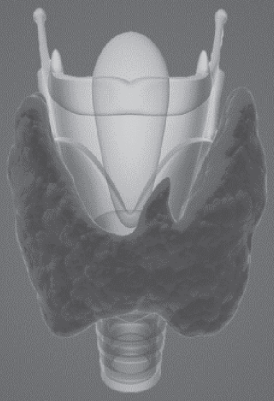
CHAPTER 9

Summary and Discussion



DISCUSSION

This thesis describes clinical and fundamental studies addressing clinical challenges in patients with differentiated thyroid carcinoma (DTC). The diagnosis of DTC is hampered by the fact that although the incidence is low thyroid nodules are prevalent. In this thesis, the diagnostic value of a potential marker for DTC has been studied. Unfortunately, in a high proportion of patients with metastases of DTC, the tumor has become resistant to RAI therapy. We have performed fundamental studies into the mechanisms of the regulation of the sodium iodide symporter (NIS) that may have implications for restoring susceptibility to RAI therapy. Furthermore, the long-term efficacy of a new treatment with the multi kinase inhibitor (MKI) sorafenib for DTC patients with metastases that are unresponsive to RAI has been evaluated. Therapy with MKI has numerous adverse effects, including hypothyroidism. We have evaluated a potential mechanism of sorafenib associated hypothyroidism. Finally, DTC patients have traditionally been treated with TSH suppressive levothyroxine substitution. However, high thyroid hormone concentrations may have adverse effects on various organ systems, including the heart. In this thesis, we studied the effects of various thyroid hormone concentrations on cardiac function in DTC, using dedicated cardiac ultrasound.

CHAPTER 2

Ultrasonography guided fine needle aspiration cytology (FNAC) is the cornerstone of the evaluation of thyroid nodules for malignancy (1). FNAC results are currently described according to the Bethesda classification (2). Indeterminate FNAC results (Bethesda class III and IV) constitute 17-30% of cases with an overall risk of malignancy of up to 30% (2). Bethesda class III and IV may correspond with follicular variant of papillary thyroid carcinoma (FVPTC), follicular thyroid carcinoma (FTC) or follicular adenoma (FA). Most of these indeterminate cases are referred for diagnostic hemithyroidectomy, but the majority of patients will have benign disease. Therefore improved preoperative diagnostic evaluation for patients with indeterminate cytologic findings on FNA is warranted. In Chapter 2, we set out to analyse the protein expression of Bone morphogenetic protein7 (BMP7) and 2 downstream proteins, Smad4 and pSmad1 in a large array of normal and pathological human thyroid tissues. We hypothesized that a differential expression would be present, which could have potential value for differential diagnosis of thyroid disorders. BMPs are important regulators

of growth and apoptosis in numerous tissues and are associated with cancer progression (3, 4). Few mechanistic studies on the BMP7 pathway have been published in thyroid (5-7); including one study in carcinoma (8). However, until now, no systematic studies on tissue expression of BMP7 and downstream proteins in normal and pathological human thyroid tissues have been performed. We used a tissue array containing 750 thyroid samples from 118 patients (38 had benign thyroid tissues lesions and 80 cases of non-medullary thyroid carcinomas (PTC, FTC, FVPTC) and mixed PTC and FTC (PTCmixFTC). Expression of the above mentioned proteins was studied by immunohistochemistry and analysed semi quantitatively by 3 observers. The differential expression was analysed by a multilevel regression model followed by Bonferroni correction for multiple testing. Normal thyroid tissue was considered as reference. In thyroid carcinoma cytoplasmic BMP-7, Smad4 and p-Smad1 expression was increased with low nuclear pSmad1 expression. This pattern was present in all malignant subtypes except for PTC where the cytoplasmic Smad4 was over-expressed. However, nuclear expression of pSmad1 (npSmad1, end downstream protein of the BMP7 signalling cascade) in all tumor types was consistently decreased Thus, BMP7 cytoplasmatic protein expression *per se* may not be necessarily associated with increased activity but may rather reflect diminished activity of the BMP7 cascade. In the analysis of follicular lesions, npSmad1 expression was lower in FVPTC compared with FA. Unfortunately, we found no differences in expression of BMP7 or downstream proteins between FTC and FA with diagnostic potential. However, the decreased npSmad1 expression in FVPTC vs. increased expression in FA may be worthwhile to explore in further studies.

Perspective

We found differentially expressed proteins of the BMP7 signalling pathway in a large array of normal, benign and malignant thyroid tissues. The underlying functional mechanisms remain to be established, whereas the diagnostic potential of our observations seems to be limited.

CHAPTER 3

Successful RAI therapy is dependent on the stimulation of iodide uptake by TSH. In terms of preparation for RAI, high TSH levels can be realized either by discontinuation of levothyroxine therapy or by the application of recombinant human TSH (rhTSH) during levothyroxine therapy (9, 10). rhTSH aided RAI therapy is based on the assumption that the

continuation of levothyroxine substitution has no effect on iodide uptake. However, this has never been verified. In Chapter 3, we performed in vitro studies to analyse whether thyroid hormone directly affects RAI uptake by thyroid cells independently of TSH. Fischer rat thyroid cells (FRTL5) were cultured with T3 (1nM, 10nM & 1μM) in the presence of TSH for 1 to 4 days. To study the possible genomic regulatory action of T3 on NIS gene expression, rat NIS promoter constructs were transiently transfected in FRTL5 cells. Steady state radioactive iodide uptake by FRTL5 was reduced by 35% at the highest concentration of T3 (1μM) after 24 and 48 hours. After 4 days of 1 nM T3 treatment, iodide uptake was reduced by 30% vs. basal. NIS protein levels in FRTL-5 cells were significantly decreased in a dose dependent manner after 4 days of T3 (1nM-1 μM). Accordingly, NIS promoter activity was significantly reduced in a dose dependent manner. In conclusion, T3 down-regulates NIS protein levels and iodide uptake in follicular thyroid cells. The T3 effects on iodide uptake, NIS protein levels and NIS promoter activity take several days to become manifest. The effects seem to be mediated by decreased NIS promoter activity.

Perspective

We found that T3 decreases NIS protein expression independently of TSH. If confirmed in humans, this may have major impact on the feasibility of RAI therapy with rhTSH, as the assumption of this therapy is that T3 in itself does not influence iodide uptake. Although in recent studies, a comparable ablation rate was found in rhTSH vs thyroid hormone withdrawal, long term outcome analyses are needed to ultimately determine the equivalence of both approaches. In addition, our studies do not support rhTSH in RAI therapy in patients with structural disease (11), although equivalence studies are need in this context as well.

CHAPTER 4

Sorafenib (BAY 43-9006) is an oral MKI targeting wild-type and mutant BRAF V600E, RET, VEGFR2, VEGFR3, PDGFR-β, c-KIT and Flt-3 (12, 13). Sorafenib has been approved by the FDA and the EMA for the treatment of metastatic RAI resistant DTC patients (14, 15). Sorafenib has been studied in a number of phase II clinical trials, one retrospective longitudinal study and 1 phase III, randomized, placebo-controlled, multicenter trial study (DECISION trial) (12, 13, 16- 21). In Chapter 4, we analysed the long-term outcome of a phase II study. Thirty-one patients with progressive metastatic or locally advanced RAI

refractory (RAIR) DTC received sorafenib 400 mg orally twice daily. The study end points included response rate, progression-free survival (PFS), overall survival (OS), best response by RECIST 1.0, and toxicity. Median PFS was 18 months and median OS was 34.5 months. Eight patients (31%) achieved a partial response and 11 patients (42%) showed stable disease after a median follow-up of 25 months. Toxicity mostly included hand and foot syndrome, weight loss, diarrhea, and rash. We conclude that sorafenib has clinically relevant antitumor activity in patients with progressive metastatic or locally advanced RAIR DTC.

Perspective

After our study was completed, the phase III DECISION with sorafenib was published (see introduction) (21). Median progression-free survival was significantly longer in the sorafenib group (10.8 months) than in the placebo group. Later, the SELECT study with lenvatinib showed an even more impressive outcome with PFS of 18.3 months in lenvatinib vs. 3.6 months in placebo (22). Both studies have for the first time shown that MKI have a benefit on PFS in patients with advanced DTC. Important questions to be answered are the selection of subgroups of patients who benefit most from these drugs and the optimal time point of initiation of therapy. These questions are prominent, as MKI are associated with considerable toxicity and subsequent diminished quality of life. It is now recommended to initiate therapy with MKI when RAI resistance is present, when progression has been established according to RECIST and when considerable tumor load is present. As the pathogenesis of RAIR DTC is multifactorial, and resistance to MKI is common, a rational approach to be investigated will be the combination of different treatment strategies. Furthermore, the use of MKI's (e.g selumetinib) in restoring RAI-uptake must be further investigated.

CHAPTER 5

Therapy with MKI in patients with malignancies is associated with alterations in thyroid hormone parameters. Sorafenib (17), imatinib (23, 24), motesanib (25), and sunitinib (26) have also been associated with thyroid function abnormalities in athyreotic patients with thyroid carcinoma. Sorafenib was associated with increased TSH levels in 33% of thyroid carcinoma patients. Because an increased need of levothyroxine is observed in athyreotic patients who are treated with MKI, we hypothesized that one of the mechanisms of hypothyroidism may include alterations in thyroid hormone metabolism. In Chapter 5 we

conducted a retrospective study that included 21 DTC patients who were receiving levothyroxine as a replacement therapy in a dose adjusted to keep the TSH levels below 0.1mU/L. They were treated with sorafenib for 26 weeks. At baseline and after 26 weeks of therapy, serum thyroid parameters were measured (T4, free T4, T3, free T3, reverse T3 (rT3), and TSH concentrations). We observed a significant increase in levothyroxine dose/kg while T4 and fT4 remained unchanged. Absolute rT3 levels tended to increase after sorafenib therapy, and T3/T4, T3/rT3, and T4/rT3 ratios decreased significantly, reflecting an increased conversion of T4 into rT3. No effects of sorafenib on thyroid hormone binding were observed. The decreased T3/T4 and T3/rT3ratios are best explained by an increased D3 activity. In this study we addressed for the first time that MKI in addition to a direct effect on the thyroid gland, also enhances peripheral metabolism of thyroid hormones, likely by enhancing activity of D3.

Perspective

TSH suppressive levothyroxine substitution is the cornerstone therapy in DTC patients with metastases. However, we demonstrate in our study that MKI therapy enhances levothyroxine metabolism. Surveillance and adjustment of levothyroxine therapy is therefore of utmost relevance in DTC patients on treatment with MKI. In a subsequent study by another group it was demonstrated that sunitinib (27), induces hypothyroidism through increase of hepatic D3 and decrease of D1 activity respectively as well as thyroid capillary regression. Interestingly, in a recent study, it was shown that MKI also decreases thyroid hormone influx in the pituitary by inhibiting MCT8 activity (28) which may be another explanation for the elevated TSH in patients on MKI.

CHAPTER 6

In Chapter 6, we performed in depth studies on the regulation of functional NIS expression, in order to identify new approaches to enhance RAI uptake in DTC. Our studies are based on the observation that AMPK plays an important role in NIS expression: AMPK activation in thyrocytes decreases iodide uptake, NIS expression and increases glucose uptake (29, 30). We examined the effect of AMPK modulators on thyrocyte function in-vitro (FRTL5 cells) and in-vivo (C57Bl6/J mice). In addition, we studied the transcriptional regulatory role of the AMPK- dependent signalling pathway on NIS protein using rat NIS promoter constructs.

Activation of AMPK by metformin resulted in a strong reduction of iodide uptake (up to six fold with 5 mM metformin after 96 h) and NIS protein levels in vitro, whereas AMPK inhibition by compound C not only stimulated iodide uptake but also enhanced NIS protein levels both in vitro (up to sevenfold with 1 μM compound C after 96 h) and in vivo (1.5-fold after daily injections with 20 mg/kg for 4 days). Metformin reduced NIS promoter activity (0.6-fold of control), whereas compound C stimulated its activity (3.4-fold) after 4 days. This largely coincided with cAMP responsive element (CRE) activation (0.6- and 3.0-fold). Apparently, the downstream effects of TSH activation and AMPK action seem to synergize on the CRE-like element of the NIS promoter. In conclusion, NIS expression and iodide uptake in thyrocytes can be modulated by metformin and compound C that exert their effects by modulating AMPK, which, in turn, regulates the activation of the CRE element in the NIS promoter.

Perspective

Enhanced iodide uptake by compound C suggests that AMPK inhibition may provide a useful mechanism for the enhancement of iodide uptake in the treatment of DTC. As AMPK has multiple effects on numerous signal transduction routes involved in metabolism, proliferation and autophagy, additional studies in DTC patients are necessary.

CHAPTER 7

Patients with DTC will be exposed to either low thyroid hormone levels, during preparation for ablation and/or therapy with RAI, or high thyroid hormone levels, when TSH suppressive levothyroxine substitution is given. As thyroid hormone plays an important role in cardiac function, we analysed the effects of TSH suppressive thyroid hormone substitution and withdrawal on cardiac function.

In Chapter 7, we analysed the effects of prolonged TSH suppressive levothyroxine therapy on cardiac dimensions and function using 2-dimensional (2D) speckle tracking echocardiography, which permits the study of multidirectional active deformation of the myocardium (radial, circumferential and longitudinal strain and strain rate), providing comprehensive information on myocardial function (contractility) (31, 32). Twenty-five patients with a history DTC on long-term TSH-suppressive levothyroxine substitution were

randomized to persistent TSH-suppressive substitution (low-TSH group) or restoration of euthyroidism. Additionally 40 euthyroid controls were studied. At baseline, DTC patients showed normal left ventricular (LV) systolic function but impaired diastolic function as assessed with conventional echocardiographic parameters. Importantly, 2D speckle tracking echocardiography demonstrated subclinical LV systolic and diastolic dysfunction with impaired circumferential and longitudinal strain and strain rate at the isovolumic relaxation time. After restoration of euthyroidism, a significant improvement in LV systolic and diastolic function as assessed with 2D- speckle tracking echocardiography was observed.

Perspective

Our study contributes to the notion that TSH suppressive levothyroxine substitution has disadvantageous effects on the heart, but that functional changes are reversible, even after prolonged exposure to TSH suppression. Given the deleterious adverse effects of TSH suppression on many organ systems, recently, recommendations regarding TSH suppression have become more risk adapted (33). As a consequence, the majority of cured DTC patients will receive levothyroxine substitution, aiming at normal TSH levels.

CHAPTER 8

During levothyroxine withdrawal for RAI therapy, patients are repeatedly exposed to short-term hypothyroidism. However, the cardiovascular effects of transitions from exogenous subclinical hyperthyroidism (TSH suppressive levothyroxine substitution) to short-term overt hypothyroidism in patients treated for DTC remain unclear. In Chapter 8, we performed a study to evaluate the changes in multidirectional myocardial strain using 2D speckle tracking echocardiography during the controlled transition from exogenous subclinical hyperthyroidism to overt hypothyroidism. The study included 14 patients with DTC on TSH-suppressive levothyroxine substitution who were subsequently withdrawn from levothyroxine for 4 weeks. Cardiac function was assessed by 2D speckle tracking echocardiography before and at 1 and 4 weeks after withdrawal and compared with values obtained in a control group. At baseline, the LV dimensions were significantly higher in patients as compared with controls. Using 2D speckle tracking echocardiography, DTC patients had significantly impaired baseline myocardial systolic function in the circumferential and longitudinal directions as compared with controls. Withdrawal of thyroid hormone did not induce

significant changes in LV dimensions or systolic function. We did not observe any change in radial strain measurements. Remarkably, during the transition from exogenous subclinical hyperthyroidism to overt hypothyroidism, a significant improvement in circumferential and longitudinal systolic shortening was observed and returned to abnormal values after 4 weeks when the patients were overtly hypothyroid. Apparently, a u-shaped relationship between a range of thyroid hormone levels (from hyper- to hypothyroid concentrations) and myocardial strain was observed.

Perspective

Using advanced cardiac ultrasound techniques, our study uncovered subtle changes in cardiac function during the course from TSH suppressive therapy to overt hypothyroidism. We found that both TSH suppressive levothyroxine therapy and hypothyroidism affect myocardial function. Although the clinical consequences of these observations are not clear, they contribute to the findings from numerous studies that DTC patients are at increased risk for cardiovascular events, which are related to levothyroxine substitution (34, 35). The risk-based tailoring of levothyroxine therapy as mentioned above as well as the introduction of rhTSH to avoid hypothyroidism may therefore be beneficial in decreasing cardiovascular risk in DTC patients.

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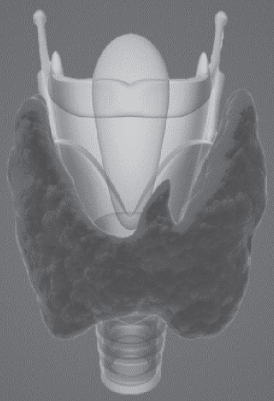
CHAPTER 10

Nederlandse samenvatting



NEDERLANDSE SAMENVATTING

Dit proefschrift beschrijft klinische en fundamentele studies welke gerelateerd zijn aan patiënten met gedifferentieerd schildklier carcinoom (DTC).

Het stellen van de juiste diagnose bij deze groep patiënten wordt bemoeilijkt omdat binnen de groep van patiënten met schildkliernodi de incidentie van patiënten met DTC laag is. Een verbetering van de diagnostiek is daarom gewenst. In dit proefschrift beschrijven wij de potentiele diagnostische waarde van een marker voor DTC.

Een belangrijk probleem bij de standaard behandeling met radioactief jodium (RAJ) is het verlies van het vermogen tot jodiumopname in progressieve tumoren en metastasen. Een verder inzicht in de mechanismen die de jodiumopname reguleren geeft wellicht aanknopingspunten voor toekomstige behandelingen. Vanuit deze optiek hebben wij op moleculair niveau enkele mechanismen onderzocht die de werking van de sodium iodide symporter (NIS) beïnvloeden.

Daarnaast hebben wij de lange termijn effecten onderzocht van de behandeling van DTC patiënten met metastasen die het vermogen tot jodiumopname verloren hebben met de multikinase remmer (MKI) sorafenib. Een van de bekende bijwerkingen van de MKI behandeling is het ontstaan van hypothyreodie. In dit proefschrift beschrijven we een mechanisme dat mogelijk ten grondslag ligt aan het ontstaan van de aldus ontstane hypothyreodie.

Binnen de conventionele behandeling van DTC patiënten wordt TSH onderdrukt door levothyroxine (schildklierhormoon) substitutie. De gebruikelijke hoge concentraties schildklierhormoon kunnen echter resulteren in nadelige effecten op verschillende organen met inbegrip van het hart. In dit proefschrift beschrijven wij de effecten van schildklierhormoon concentraties op de hartfunctie van DTC patiënten met behulp van cardiale echografie.

HOOFDSTUK 2

Echogeleide fijne naald aspiratie cytologie (fine needle aspiration cytology (FNAC)) neemt een centrale plaats in bij het diagnosticeren van maligniteit bij schildkliernodi waarbij de uitkomsten van FNAC worden geclassificeerd via het Bethesda system. Bij 17-30% van de

patiënten is de uitslag van FNAC bethesda klasse III of IV met een kans van 30% op maligniteit. Gezien de onzekere diagnose zullen patiënten binnen deze groepen (die o.a. folliculaire variant van papillair schildkliercarcinoom (FVPTC), folliculair schildkliercarcinoom (FTC) en folliculair adenoom (FA) bevatten) vaak worden doorverwezen voor diagnostische hemithyreoïdectomie ondanks het feit dat de meerderheid van de nodi goedaardig zal blijken te zijn. Een verbetering in de diagnostiek bij deze patiënten na FNAC is dan ook gewenst.

In hoofdstuk 2 hebben wij met behulp van een tissue array met een groot aantal pathologische en normale humane schildklierweefsels de eiwitexpressie geëvalueerd van Bone morphogenetic protein7 (BMP7), Smad4 en pSmad1. Hierbij was onze hypothese dat er een verband bestaat tussen de aard van de nodi en de differentiële expressie van deze eiwitten. BMP's reguleren de groei en apoptose in talloze weefsels en worden in verband gebracht met progressie van tumoren. Er zijn echter maar enkele mechanistische studies verricht naar de rol van BMP's in de schildklier en tot nu toe zijn er geen systematische studies gedaan naar BMP7 expressie in normale en pathologische weefsels.

Wij hebben gebruik gemaakt van een tissue array met 750 schildklier monsters afkomstig van 118 patiënten met goedaardige en kwaadaardige schildkliertumoren. (38 benigne, 80 niet medullair schildkliercarcinoom (PTC, FTC, FVPTC) en gemengd PTC/ FTC (PTCmixFTC)). Na een immunohistologische bepaling zijn de expressie niveaus geanalyseerd met een multilevel regressie model gevolgd door een Bonferroni correctie met normale schildklier weefsels als referentie.

De cytoplasmatische expressie niveaus van BMP7, Smad4 en pSmad1 waren verhoogd in alle maligne subtypes met uitzondering van PTC waar alleen de expressie van Smad4 was verhoogd. De nucleaire expressie van pSmad1(npSmad1, downstream van BMP7) nam echter af in alle maligne weefsels wat erop duidt dat de cytoplasmatische toename van BMP7 wellicht geassocieerd kan worden met een afgenomen activiteit van de BMP7 pathway.

De diagnostische mogelijkheden om te differentiëren tussen FTC en FA met behulp van BMP7, Smad4 en pSmad1 expressie analyse lijken helaas beperkt aangezien wij geen verschillen konden vinden tussen FTC en FA. Alleen in de subgroep FVPTC vs. FA vonden wij een verschil in npSmad1 expressie (in FVPTC lager dan in FA).

Perspectief

Wij hebben differentiële expressie gevonden bij eiwitten van de BMP7 pathway in een tissue array met normaal schilklierweefsel, goedaardige en kwaadaardige schildkliertumoren. Aangezien differentiatie tussen FTC en FA helaas niet mogelijk was concluderen wij dat BMP7, Smad4 en pSmad1 niet geschikt zijn voor de differentiaal diagnose tussen FTC en FA.

HOOFDSTUK 3

Succesvolle RAJ therapie is afhankelijk van de stimulatie van jodide opname door TSH. Voorafgaand aan RAJ kunnen hoge TSH niveaus worden gerealiseerd door stopzetting van de levothyroxine therapie of door toediening van recombinant humaan TSH (rhTSH) tijdens de levothyroxine therapie. Deze praktijk is gebaseerd op de veronderstelling dat voortzetting van levothyroxine substitutie geen nadelig effect heeft op de jodide opname, hoewel dit nooit is aangetoond. In hoofdstuk 3 beschrijven wij in vitro experimenten waarin wij de directe invloed van schildklierhormoon analyseren op RAJ opname door folliculaire schildkliercellen. Fischer rat schildkliercellen (FRTL5) werden gecultiveerd in aanwezigheid van T3 (1 nM, 10 nM en 1 uM) en TSH gedurende 1 tot 4 dagen. Om de mogelijke genomische regulatie van NIS expressie door T3 te bestuderen hebben wij verscheidene NIS promoter constructen getransfecteerd in FRTL-5 cellen.

Steady state RAJ opname door FRTL5 nam af met 35% bij de hoogste T3 concentratie (1 uM) na 24 en 48 uur. Na 4 dagen was er ook een afname in jodide opname bij 1 nM T3 (30% vs. basaal). De hoeveelheid NIS eiwit in FRTL-5 cellen nam eveneens af op een dosisafhankelijke wijze na 4 dagen T3 (1nM- 1uM) behandeling. Deze effecten komen overeen met effecten op de NIS promoter.

Perspectief

Uit onze studies blijkt dat T3 de jodium opname door NIS en de NIS eiwit expressie in FRTL-5 cellen kan onderdrukken, onafhankelijk van TSH. Indien dit mechanisme ook een rol speelt in de humane schildklier kan dit van invloed zijn op het gebruik van RAJ therapie in combinatie met levothyroxine. In recente studies kon echter geen relatie worden aangetoond tussen de effectiviteit van RAJ en levothyroxine behandeling, hoewel lange termijnstudies nodig zijn.

HOOFDSTUK 4

Sorafenib (BAY 43-9006) is een oraal toegediende MKI die aangrijpt op BRAF V600E, RET, VEGFR2, VEGFR3, PDGFR-β, c-KIT en Flt-3. Sorafenib is goedgekeurd door de FDA en de EMA voor de behandeling van DTC patiënten met RAJ ongevoelige uitzaaiingen. Sorafenib is onderzocht in een aantal klinische fase II studies, een retrospectieve longitudinale studie en een fase III, gerandomiseerde, placebo-gecontroleerde, multicenter studie (DECISION trial). In hoofdstuk 4 analyseren wij de lange termijn resultaten van een fase II studie.

Eenendertig DTC patiënten met progressief gemetastaseerd of RAJ refractair gedifferentieerd schildkliercarcinoom kregen tweemaal daags 400 mg sorafenib. De studie eindpunten waren progressie-vrije overleving (PFS), algemene overleving (OS), beste respons volgens RECIST 1.0 en toxiciteit. De mediane PFS was 18 maanden en de mediane OS was 34,5 maanden. Acht patiënten (31%) hadden een partiële respons en bij 11 patiënten (42%) bleef de ziekte stabiel na een mediane follow-up van 25 maanden. Bijwerkingen bestonden uit hand en voet syndroom, gewichtsverlies, diarree en huiduitslag. Wij concluderen dat sorafenib klinisch relevante anti-tumor activiteit heeft bij DTC patiënten met RAJ resistente progressieve metastasen.

Perspectief

Nadat onze studie was afgerond is de fase III DECISION met sorafenib gepubliceerd. Progressie-vrije overleving was significant langer in the sorafenib group (mediaan 10.8 maanden) in vergelijking met de placebo groep. De SELECT studie met lenvatinib bleek later een nog indrukwekkender resultaat te geven met een PFS van 18.3 maanden vs. 3.6 maanden in de placebogroep. Beide studies hebben voor de eerste keer aangetoond dat MKI's bruikbaar kunnen zijn bij de behandeling van patiënten met gevorderde DTC. Belangrijke vragen die nog beantwoord moeten worden zijn gerelateerd aan de respons bij deelgroepen van patiënten en het optimale tijdstip voor aanvang van de therapie. Deze vragen zijn met name van belang omdat MKI's geassocieerd worden met aanzienlijke toxiciteit en de daaruit voortvloeiende verminderde kwaliteit van leven. Aanbevolen wordt om MKI therapie te starten bij RAJ resistentie, wanneer progressie is vastgesteld volgens RECIST en wanneer er een aanzienlijke tumor belasting aanwezig is. Aangezien de pathogenese van RAJ resistentie van vele factoren afhangt en MKI resistentie veelvuldig voorkomt zal een rationele benadering moeten bepalen

welke combinatie van behandelingsstrategieën wenselijk is. Verder is het zeker de moeite waard om de mogelijkheden van MKI therapie te onderzoeken in hoog risico patiënten die hun vermogen tot RAJ opname nog niet verloren hebben.

HOOFDSTUK 5

MKI behandeling van patiënten met maligniteiten wordt geassocieerd met veranderingen in schildklier hormoon parameters. Verder zijn sorafenib, imatinib, motesanib, and sunitinib in verband gebracht met afwijkingen van de schildklierfunctie in patiënten met schildkliercarcinoom die geen schildklier meer hebben. Na behandeling met sorafenib stegen TSH niveaus in 33% van de schildkliercarcinoom patiënten. Aangezien een toegenomen behoefte aan levothyroxine wordt waargenomen bij patiënten zonder schildklier die zijn behandeld met MKI, postuleerden wij de hypothese dat een van de mechanismen voor het ontstaan van hypothyreoidie wellicht te herleiden zou zijn tot veranderingen in het schildklierhormoon metabolisme.

In hoofdstuk 5 beschrijven wij een retrospectieve studie met 21 DTC patiënten die levothyroxine therapie ontvingen, bij wie het doel was om de TSH niveaus beneden 0.1mU/L te houden. Deze patiënten werden gedurende 26 weken behandeld met sorafenib.

Bij aanvang en na 26 weken behandeling werden serum schildklier parameters gemeten (T4, vrij T4, T3, vrije T3, reverse T3 (rT3), en TSH concentraties) waarbij wij een significante toename zagen in de dosis levothyroxine (dosis/kg) terwijl T4 en fT4 stabiel bleven. Absolute rT3 niveaus hadden de neiging om na sorafenib therapie toe te nemen terwijl T3/T4, T3/rT3, en T4/rT3 verhoudingen aanzienlijk daalden wat wijst op een verhoogde omzetting van T4 naar rT3. Sorafenib lijkt geen invloed te hebben op schildklierhormoon binding en de afname in T3/T4 enT3/rT3 ratios kunnen het beste verklaard worden door een verhoogde activiteit van het enzym deiodinase type 3 (D3).

Perspectief

TSH suppressieve levothyroxine substitutie staat centraal bij de therapie van DTC patiënten met metastasen. Uit onze studie blijkt dat MKI therapie het levothyroxine metabolisme verhoogt. Daarom is het van cruciaal belang dat bij DTC patiënten die worden behandeld met MKI sterk gelet wordt op de benodigde levothyroxine dosis. Bij een andere studie met

sunitinib is onlangs aangetoond dat deze MKI hypothyreoidie induceert door een toename van hepatisch D3 en een afname van D1 activiteit. In een recente studie werd onlangs aangetoond dat MKI de schilklierhormoon influx in de hypofyse beperkt door remming van MCT8 activiteit. Dit zou nog een aanvullende verklaring kunnen zijn voor de verhoogde TSH waarden in patiënten die worden behandeld met MKI.

HOOFDSTUK 6

Er zijn aanwijzingen dat jodide opname en functionele NIS expressie worden gereguleerd door AMP kinase (AMPK) activatie. Deze recente bevindingen leveren wellicht nieuwe aangrijpingspunten voor de verbetering van RAJ behandeling in DTC patiënten. Wij hebben de effecten onderzocht van AMPK modulatoren op de functie van thyrocyten, in vitro (FRTL-5 cellen) en in vivo (C57B16/J muizen). Daarnaast hebben we onderzocht of de effecten op jodide opname en functionele NIS expressie door AMPK op transcriptioneel niveau gereguleerd worden met behulp van NIS promoter luc-reporter constructen. In vitro leidde activatie van AMPK door metformine tot een sterke afname van jodide opname (6-voudige afname na 96h, 5mM metformine) en NIS eiwit. AMPK remming door CompoundC stimuleerde de jodiumopname in vitro (7-voudige toename na 96h, 1 μM compound C) en in vivo (1.5x na dagelijkse injecties gedurende 4 dagen met 20 mg/kg). De NIS promoter activiteit nam af na metformine behandeling (0.6x) terwijl compoundC de NIS promoter stimuleerde (3.4x) na 4 dagen. Dit correspondeert grotendeels met cAMP responsive element (CRE) activatie (0.6x en 3.0x) wat impliceert dat TSH en AMPK beide aangrijpen op het CRE element in de NIS promoter. Concluderend kunnen we stellen dat de NIS expressie en jodide opname in thyrocyten gemoduleerd kunnen worden door metformine en compoundC, waarbij regulatie van het CRE element via AMPK in de NIS promoter een rol speelt

Perspectief

De toename van jodiumopname na toediening van compoundC duidt erop dat AMPK remming interessante aanknopingspunten biedt voor de verbetering van jodide opname bij de behandeling van DTC. Additionele studies zullen moeten uitwijzen of AMPK een valide target is zeker gezien het feit dat AMPK vele andere pathways kan aansturen.

HOOFDSTUK 7

Patiënten met DTC worden zowel blootgesteld aan lage (ter voorbereiding op RAJ) als hoge schildklierhormoon waarden (TSH onderdrukkende levothyroxine substitutie). Aangezien schildklierhormoon een belangrijke rol speelt bij de cardiale functie hebben wij de effecten van deze hoge en lage schildklierhormoonwaarden bij DTC patiënten op cardiale dimensies en functie onderzocht met behulp van 2-dimensionale (2D) speckle tracking echocardiography. Deze methode maakt het mogelijk om het myocard te bestuderen in meerdere dimensies (radial, circumferential en longitudinal strain en strain rate), zodat uitgebreide informatie wordt verkregen over de myocardiale functie (contractiliteit).

Vijfentwintig patiënten met een verleden van DTC en langdurige TSH suppressieve levothyroxine substitutie werden gerandomiseerd en onderverdeeld in een groep met onderdrukte TSH (lage TSH groep) en een waarbij euthyroidie werd hersteld en vergeleken met 40 controles met euthyreoidie.

Bij aanvang vertoonden DTC patiënten een normale linker ventrikel (LV) systolische functie maar een verminderde diastolische functie volgens conventionele echocardiografische parameters. Met behulp van 2D speckle tracking echocardiography hebben wij subklinische LV systolische en diastolische dysfunctie aangetoond waarbij een verminderde circumferential en longitudinal strain en strain rate waarneembaar waren tijdens de isovolumetrische relaxatie tijd. Na herstel van euthyreoïdie werd een significante verbetering in LV systolische en diastolische functie waargenomen.

Perspectief

Onze studie onderschrijft dat TSH suppressieve levothyroxine substitutie nadelige effecten heeft op het hart. Deze functionele veranderingen zijn echter reversibel, zelfs na langdurige blootstelling aan TSH suppressie. Gezien de nadelige effecten van TSH suppressie op vele orgaansystemen zijn recent de aanbevelingen betreffende TSH suppressie aangepast. Bijgevolg zal de meerderheid van de behandelde DTC patiënten een levothyroxine substitutie krijgen die gericht is op normale TSH waarden.

HOOFDSTUK 8

Tijdens levothyroxine onttrekking gedurende RAJ therapie worden patiënten herhaaldelijk blootgesteld aan korte perioden van hypothyreoïdie. De cardiovasculaire effecten van deze

overgangen tussen exogene subklinische hyperthyreoïdie (TSH-suppressieve levothyroxine substitutie) naar korte termijn hypothyreoïdie in patiënten behandeld voor DTC waren tot dusverre onbekend. In hoofdstuk 8 beschrijven we een studie naar de veranderingen in multidirectionele myocardial strain met behulp van 2D-speckle tracking echocardiografie gedurende de transitie van exogene subklinische hyperthyroïdie naar openlijke hypothyroïdie. Van 14 DTC patiënten die onder behandeling stonden met TSH-suppressieve levothyroxine therapie werd gedurende 4 weken de levothyroxine behandeling stopgezet. Van deze groep werd de hartfunctie beoordeeld met behulp van 2D speckle tracking-echocardiografie vóór en 1 en 4 weken na onttrekking van levothyroxine waarna deze werden vergeleken met waarden uit een controlegroep.

DTC patiënten bleken een aanzienlijk verminderde circumferential en longitudinal strain te hebben ten opzichte van de controle groep. Onttrekking van schildklierhormoon heeft geen significante veranderingen in de linker ventrikel dimensies of systolische functie tot gevolg. Met behulp van 2D speckle tracking echocardiografie hebben wij geen veranderingen waargenomen in radial strain. Opvallend was dat tijdens de transitie van exogene subklinische hyperthyreoïdie naar openlijke hypothyreoïdie een aanzienlijke verbetering werd waargenomen in omtrek en longitudinale systolische verkorting. Na 4 weken en het bereiken van hypothyroïdie verslechterden de parameters weer. Het lijkt erop dat er een u-vormige relatie bestaat tussen schildklier hormoon niveaus en myocardial strain.

Perspectief

Met behulp van geavanceerde cardiale echografie technieken heeft onze studie subtiele veranderingen in de hartfunctie aangetoond tijdens de overgang van TSH suppressieve therapie naar openlijke hypothyreoïdie. We vonden dat zowel TSH suppressieve levothyroxine therapie als hypothyreoïdie de myocardiale functie kunnen verminderen. Hoewel de klinische consequenties van deze vindingen onbekend zijn dragen ze bij aan de bevindingen uit tal van studies die laten zien dat DTC patiënten een verhoogd risico hebben op cardiovasculaire problemen, die gerelateerd zijn aan levothyroxine substitutie. Het nauwkeurig afstemmen van de levothyroxine therapie en de toediening van rhTSH in plaats van schildklierhormoon onttrekking om hypothyreoïdie voorkomen zal wellicht bijdragen aan het verminderen van het cardiovasculair risico in DTC patiënten.

CURRICULUM VITAE

Randa Mostafa Abdulrahman Hareedy was born in the 25th of November 1975 in Assiut, Egypt. In 1992 she was awarded her high school diploma from Khadijah Yusuf High School for girls, Assiut, Egypt (A+ level). Between 1992 and 1999 the author studied medicine in the Faculty of Medicine, Assiut University, Egypt where she graduated after finishing her internship year (cum laude).

In May 2000 the author began her professional carrier as a Demonstrator Medical Biochemistry in the department of Medical Biochemistry, Assiut University Faculty of Medicine, Egypt. In the period between 2001 and 2003 the author had a position as clinical research fellow in the Endocrinology Unit, Internal Medicine Department, Assiut University Hospital. In 2005 she obtained her Master degree in Medical Biochemistry from Assiut University after defending her Master thesis "Renal tubular dysfunction in patients with non-insulin dependent diabetes mellitus" under supervision of professors Dr. Thorya Eldeeb and Dr. Nagla Elmelegy. From January 2006 she has been working as an Associate Lecturer of Medical Biochemistry. Since 2001 the author was involved in clinical and basic research, medical education and clinical and chemical pathology diagnostic tasks with emphasis on Endocrinology.

Between 2008 and 2013 the author worked as a clinical research fellow in Leiden University Medical Center, the Netherlands. Currently the author is still affiliated with and working for the department of Medical Biochemistry, Assiut University Faculty of Medicine, Egypt as an Associate Lecturer of Medical Biochemistry and Chemical Pathologist in Assiut University Hospital, Egypt.

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STELLINGEN

behorend bij proefschrift

Clinical and Molecular studies in Differentiated thyroid carcinoma management

Randa Hareedy

1 Efficacy of therapy with radioactive iodide in thyroid carcinoma patients may be diminished when these patients are also being treated with metformin for diabetes mellitus (this thesis).

2 Co-administration of levothyroxine may hamper the efficacy of radioactive iodide in thyroid carcinoma patients (this thesis).

3 Multikinase inhibitors decrease serum thyroid hormone levels by multiple mechanisms, including enhanced thyroid hormone degradation (this thesis).

4 The profound effects of modulating adenosine monophosphate kinase (AMPK) on the functional expression of the sodium iodide symporter expression (NIS) demonstrate the important interplay between metabolism and oncology (this thesis).

5 The worldwide increasing incidence in thyroid carcinoma in South Korea illustrates that the introduction of novel diagnostic methods without adequate analysis of benefit and harm may lead to overdiagnosis- and treatment of malignancies (Ahn HS, Kim HJ, Welch HG. Korea's thyroid-cancer "epidemic"--screening and overdiagnosis. N Engl J Med. 2014 6;371(19):1765-7)

6 Considering thyroid carcinoma a ‘good cancer’ is harmful for patients, not only for psychological reasons, but also as it may lead to undertreatment (‘Goede kanker bestaat niet’, Anke van Haften, uitgeverij de Graaff, 2015).

7 The fact that intensive lowering of blood glucose levels in type 2 diabetes mellitus has been associated with increased mortality challenges traditional concepts in endocrinology. (Hempe JM, Liu S, Myers L, McCarter RJ, Buse JB, Fonseca V. The Hemoglobin Glycation Index Identifies Subpopulations With Harms or Benefits From Intensive Treatment in the ACCORD Trial. Diabetes Care. 2015 Apr 17. pii: dc141844)

8 Endocrinology should shift its ambitions and goals from optimizing hormone substitution therapy to developing curative therapies based on the underlying

pathophysiology of endocrine disorders (Romijn JA, Smit JW, Lamberts SW. Intrinsic imperfections of endocrine replacement therapy. Eur J Endocrinol. 2003;149(2):91-7).

9 Science, my lad, is made up of mistakes, but they are mistakes which it is useful to make, because they lead little by little to the truth. (Jules Verne, 1828 – 1905)

10 Life is like riding a bicycle. To keep your balance, you must keep moving. (Albert Einstein)

11 The more you know, the more you realise how much you don't know — the less you know, the more you think you know. (David T. Freeman)