Clinical Factors Affecting Thyroid Hormone Action and Treatment Outcome of Thyroid Diseases

Carolien M. Beukhof

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Clinical Factors Affecting Thyroid Hormone Action and Treatment Outcome of Thyroid Diseases

Klinische factoren van invloed op schildklierhormoonactie en de behandeluitkomst van schildklierziekten

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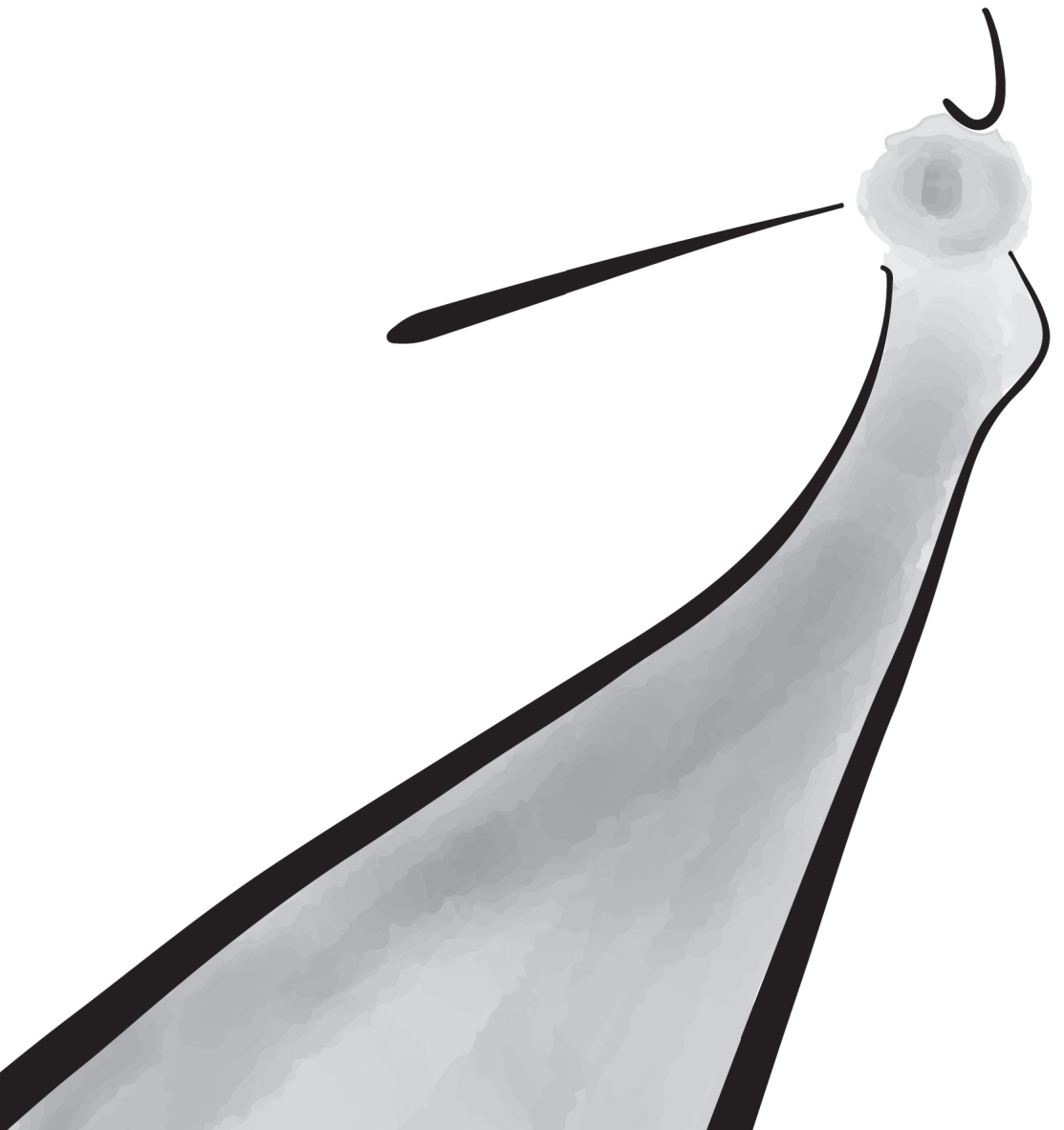

CHAPTER 1

General introduction and aims of the thesis

GENERAL INTRODUCTION

Thyroid hormones (TH) are crucial for the function of almost every organ system in the human body. Thyroid disorders are one of the most common endocrine diseases. Too little TH (hypothyroidism) results in fatigue, weight gain, cold intolerance, constipation, myopathy and also dyslipidemia and changes in renal function. Too much TH secretion (hyperthyroidism) can result in weight loss, heat intolerance, frequent bowel movements and psychological changes (1). Both severe hypo- and hyperthyroidism can eventually result in death if untreated (1). Also subclinical changes in thyroid function are associated with adverse clinical effects such as atrial fibrillation (2), osteoporosis (3), dyslipidemia (4) and atherosclerosis (5). In pregnancy subclinical TH disturbances are associated with pregnancy complications such as an increased risk of prematurity and preeclampsia (6) as well as a lower offspring IQ (7).

Hypothalamic-pituitary-thyroid axis

Serum TH levels are regulated within a narrow window. TH synthesis by the thyroid gland is controlled by the hypothalamic-pituitary-thyroid axis (HPT-axis) (Figure 1).

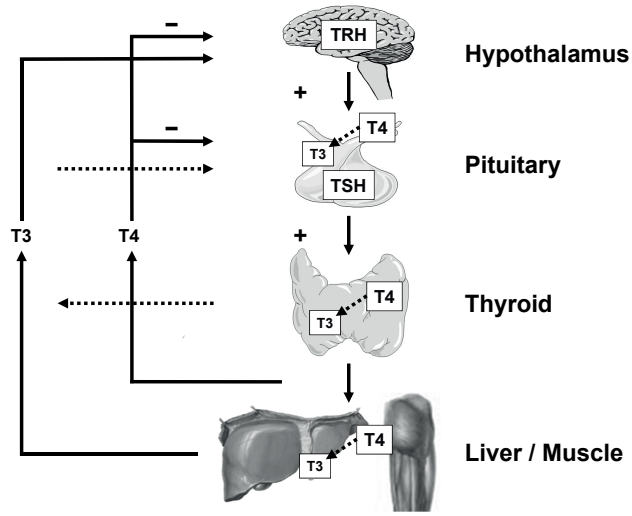

FIGURE 1

Hypothalamic release of thyrotropin-releasing hormone (TRH) results in subsequent release of thyroid stimulating hormone (Thyrotropin, TSH) by the anterior pituitary. TSH binding to the TSH receptor (TSHR), a G protein-coupled receptor, activates the cyclic Adenosine MonoPhosphate (cAMP) pathway and stimulates TH production and secretion by the thyroid gland (8). However, TSHR have been shown in skeletal muscle, bone, fat tissue and liver, indicating that TSH can also have extrathyroidal effects (9-11).
Negative feedback on the HPT-axis is dependent on intracellular T3 levels in the pituitary and hypothalamus, which is mainly derived by local conversion of T4 to T3 by type 2 deiodinase (D2) in these tissues (12, 13) (see paragraph thyroid hormone metabolism). Somatostatin also exploits an inhibitory role in the TSH secretion by pituitary. Somatostatin analogues have been found to reduce serum TSH (14).

Thyroid hormones and regulation of thyroid hormone action

Thyroid hormone metabolism

TH is composed of an inner and outer phenyl ring with iodine atoms attached at the 3, 3', 5 and 5' positions. Therefore, adequate intake of iodine via food is essential for normal TH production. Thyroxine (T4) is produced by the thyroid gland and has four iodine atoms. Removal of one iodine atom from the outer phenyl ring by intracellularly localized deiodinases results in activation of TH to triiodothyronine (T3), whereas removal of an iodine from the inner ring results in the production of the inactive metabolite reverse triiodothyronine (rT3) (15) (Figure 2).
T3 is the main biologically active TH and has only one iodine atom in the outer phenyl ring. The thyroid predominantly produces T4 and only a small percentage of total circulating T3. Circa 20% of serum T3 is derived from thyroidal secretion of T3, whereas the remaining 80% is produced by peripheral tissues such as the liver (peripheral TH metabolism). T4 can be activated into T3 by deiodinase type 1 and type 2 (D1 and D2), whereas inactivation of TH is mediated mainly via degradation of T4 and T3 by deiodinase type 3 (D3) into rT3 and diiodothyronine (T2)(16).
All three deiodinases contain a selenocysteine group in their catalytic centre and therefore belong to the class of selenoproteins. Selenoproteins are not only important in TH homeostasis but are also important in antioxidant defence (16, 17). For the biosynthesis of selenoproteins, selenium (Se), a nutritional trace element, is essential. Patients with severely compromised selenoprotein biosynthesis or massive Se deficiency show a variety of

endocrine defects including abnormal thyroid function and delayed or impaired bone formation (18-20).

Many conditions, including long-term subclinical hyperthyroidism (21) and critical illness (22), influence peripheral thyroid hormone metabolism. Also several drugs, such as corticosteroids and amiodarone, affect the activity of deiodinases (16). Furthermore, thyrosine kinase inhibitors (TKI), which are currently used to treat hepatocellular carcinoma (HCC), differentiated thyroid carcinoma (DTC) and medullary thyroid carcinoma (MTC), have been implicated to alter peripheral TH metabolism (23).

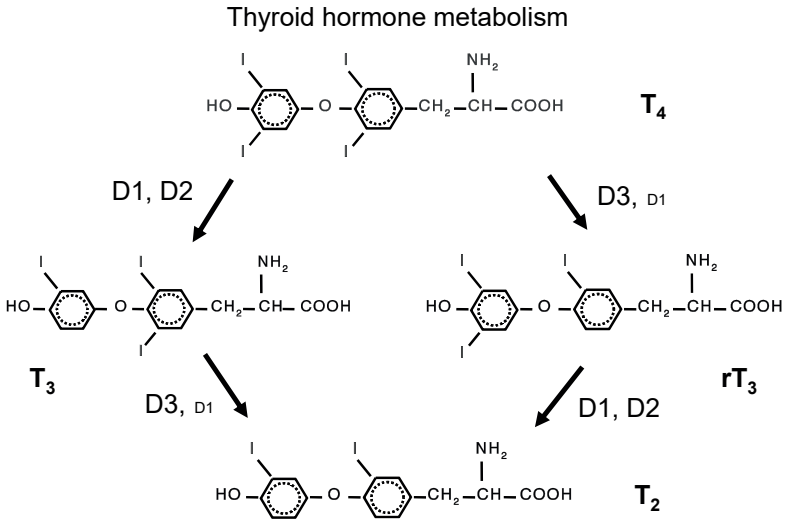

FIGURE 2

Thyroid hormone receptors

TH action is mediated via T3 receptors in the nucleus (15). Two thyroid hormone receptors (TR) with different isoforms have been identified, of which TR-α1, TR-β1 and TR-β2 are the T3 binding isoforms. These TRs have a tissue specific distribution. TR-α1 is mainly expressed in the heart, brain, skeletal muscles and gastrointestinal tract. TR-β1 is predominantly expressed in brain, liver and kidney, whereas TR-β2 has a more restrictive expression pattern in hypothalamus, pituitary and retina. TR selective agents have been developed in an attempt to mediate tissue-specific actions of TH.

Thyroid hormone transporters

The intracellular concentration and subsequent receptor binding of T3 is influenced by several mechanisms. The in- and efflux of TH over the cell membrane depends on serum concentrations of T3 and T4 and the activity of TH transporters, such as monocarboxylate transporters 8 and 10 (MCT8 and MCT10) and organic anion transporting polypeptide 1C1 (OATP1C1) (24). MCT8 is expressed in neurons in the brain and is the main transporter of the blood-brain barrier (25). MCT8 is also expressed in several other tissues including the liver, the kidney and the heart (26). Mutations in MCT8 result in the Allan-Herndon-Dudley syndrome, which is characterized by a severe neurological syndrome of X-linked mental retardation and peripheral thyrotoxicosis. Patients have low serum T4, elevated serum T3 levels and a normal or slightly elevated TSH (27). MCT10 is highly expressed in the skeletal muscles, kidney, pancreas and intestine (28). It appears to facilitate T3 uptake and efflux more than T4 (29). The clinical relevance of MCT10 remains to be established, since no patients with mutations have been identified. OATP1C1 facilitates T4 uptake into the astrocytes in the brain, and with subsequent conversion into T3 by D2 (30).

Treatment of thyroid hormone disorders

Hyperthyroidism

Excess of TH secretion by the thyroid results in the clinical syndrome "hyperthyroidism". The most common cause of primary hyperthyroidism is Graves' disease, which is an autoimmune disease caused by stimulating TSH receptor antibodies (TSHR-Ab). Treatment consists of antithyroid drugs and in case of relapse, radioactive iodine treatment is often used successfully in patients without Graves' ophthalmopathy. Surgery is reserved for patients with high risk eye disease, but is rarely necessary (31).

Other common causes of primary hyperthyroidism are toxic multinodular goiter and toxic adenoma. Due to high relapse risk after discontinuation of antithyroid drugs, radioactive iodine therapy is the first line treatment for these patients. In contrast, in patients with thyroiditis, another cause of hyperthyroidism, only requires supportive care with β blockers and painkiller, as the thyrotoxicosis spontaneously resolves in most patients (31).

Hypothyroidism

Hypothyroidism on the other hand is characterized by low TH levels. In most patients with primary hypothyroidism, the disease is irreversible due to

autoimmune destruction of the thyroid (Hashimoto's disease) and lifelong TH replacement therapy is required. Oral administration of synthetic thyroxine, levothyroxine (LT4) is the standard treatment and titrated to normalize serum TSH in order to ameliorate symptoms.
Subgroups of patients with hypothyroidism have residual symptoms despite TH replacement therapy (32). Due to a lack of endogenous T3 production by the thyroid, substitution with LT4 monotherapy generally results in higher free T4 (FT4) and lower free T3 (FT3) levels and consequently altered T3/T4 ratios compared to the healthy population (33). TH transporters and intracellular deiodinases demonstrate tissue specific regulation, therefore LT4 monotherapy may not restore euthyroidism in all peripheral tissues (34). However, at present, combined LT4 and T3 treatment continues to be a matter of debate and the beneficial effects of LT4 and T3 combinations have not been demonstrated (35).

Thyroid carcinoma

Differentiated thyroid cancer

DTC is the most frequent endocrine malignancy and can be treated by thyroidectomy and subsequent radioactive iodine ^{131}I ablation (36). Consequently, these patients completely lack remaining functional thyroid tissue and thyroidal T4 and T3 secretion. As patients are usually treated with LT4 monotherapy, serum T3 in these patients is derived from the conversion of T4 in peripheral tissues. During follow-up of DTC, patients undergo a recombinant human TSH (rhTSH) stimulation test as part of a dynamic risk stratification.
In patients with progressive metastasized disease who become refractory for radioactive iodine treatment, TKIs, such as sorafenib and lenvatinib, can be used for treatment (37). TKIs have antiangiogenic, antiproliferative and proapoptotic effects via multiple effector mechanisms, such as inhibition of the vascular endothelial growth factor receptor (38). It has been reported that LT4 treatment needs to be increased in hypothyroid patients treated with TKIs, due to an increase in TSH levels. This is presumably caused by enhanced peripheral degradation of TH by D3 (23).

Medullary thyroid carcinoma

MTC, originating from calcitonin (CT)-producing parafollicular C cells, is a rare form of thyroid cancer that accounts for less than 5% of thyroid carcinomas (39). Limited systemic treatment options are available for locally irresectable

tumor and/or distant metastases (40, 41). TKIs such as vandetanib and cabozantinib, improve progression-free survival (PFS), but unfortunately not overall survival (OS). However, grade 3 or 4 adverse events occur in 33-44% of TKI treated patients (41, 42).

AIMS OF THE THESIS

The aim of this thesis has been to study clinical factors affecting TH action as well as its consequences. In addition, we have explored a novel therapeutic modality for medullary thyroid carcinoma.

TH hormone metabolism is affected by many clinical factors. An altered metabolism of TH due to low Se status has been proposed as a mechanism for the age-dependent changes in thyroid parameters (16, 43-49). In 2012, a positive association between Se status and bone mineral density (BMD) was described in postmenopausal women (45). No data on the association of Se status and BMD in elderly men were available at that time. For that reason, we have investigated the association between Se status, thyroid function tests (TFT) (e.g. TSH, FT4, T3, rT3) and BMD in a population of 378 elderly men (**chapter 2**).

The pathogenesis of TKI-induced changes in TH levels and metabolism has not been fully elucidated (50, 51). Therefore in **chapter 3** we aimed to further unravel the effects of sorafenib on different aspects of TH homeostasis, such as direct effects on the thyroid, sensitivity of the HPT-axis and peripheral TH metabolism, by performing detailed TFT in 57 patients with HCC, as well as *in-vitro* cellular T3-uptake experiments.

The TSHR has not only been found on the surface of thyrocytes but also in a variety of other cell types including adipocytes (52). Epidemiological (5, 53-58) and experimental studies (10, 11) suggest that TSH has direct effects on serum lipids. Although TSH has been shown to increase hepatic conversion of T4 to T3 in perfused rat liver (59), direct effects of TSH on peripheral TH metabolism have not been reported in humans. To address this, we have examined the effect of exogenous rhTSH on serum lipids and peripheral TH metabolism in 81 patients, on stable LT4 maintenance doses, who had total thyroidectomy and radioactive ^{131}I treatment for of DTC (**chapter 4**).

Urinary concentration studies in hypothyroidism have been compared to healthy controls but paired analyses within the same patient are lacking (60). In **chapter 5** we have investigated the effects of short term severe hypothyroidism on urine concentration ability of the kidney, in nine patients with hypothyroidism, before and after withdrawal of LT4 treatment.

Serum TSH is currently the best available marker in patients with hypothyroidism to titrate LT4 dosing. However, TSH reflects euthyroidism at the pituitary level, but may not be representative for other target tissues. Therefore, new markers are warranted. MicroRNAs (miRNAs) are non-coding RNA molecules that show a tissue-specific expression. In **chapter 6** we have investigated the changes in miRNA profiles in hypothyroid patients on and off LT4 administration.

For progressive metastatic MTC, treatment with TKI result in grade 3-4 adverse events in a large number of patients. Peptide receptor radionuclide therapy (PRRT) with ^{177}Lu-octreotate, targeting the carcinoma mainly via the somatostatin receptor 2 (SSTR2), might be a rational therapeutic approach (61). In **chapter 7**, we have systematically analyzed the results of PRRT in ten patients with MTC.

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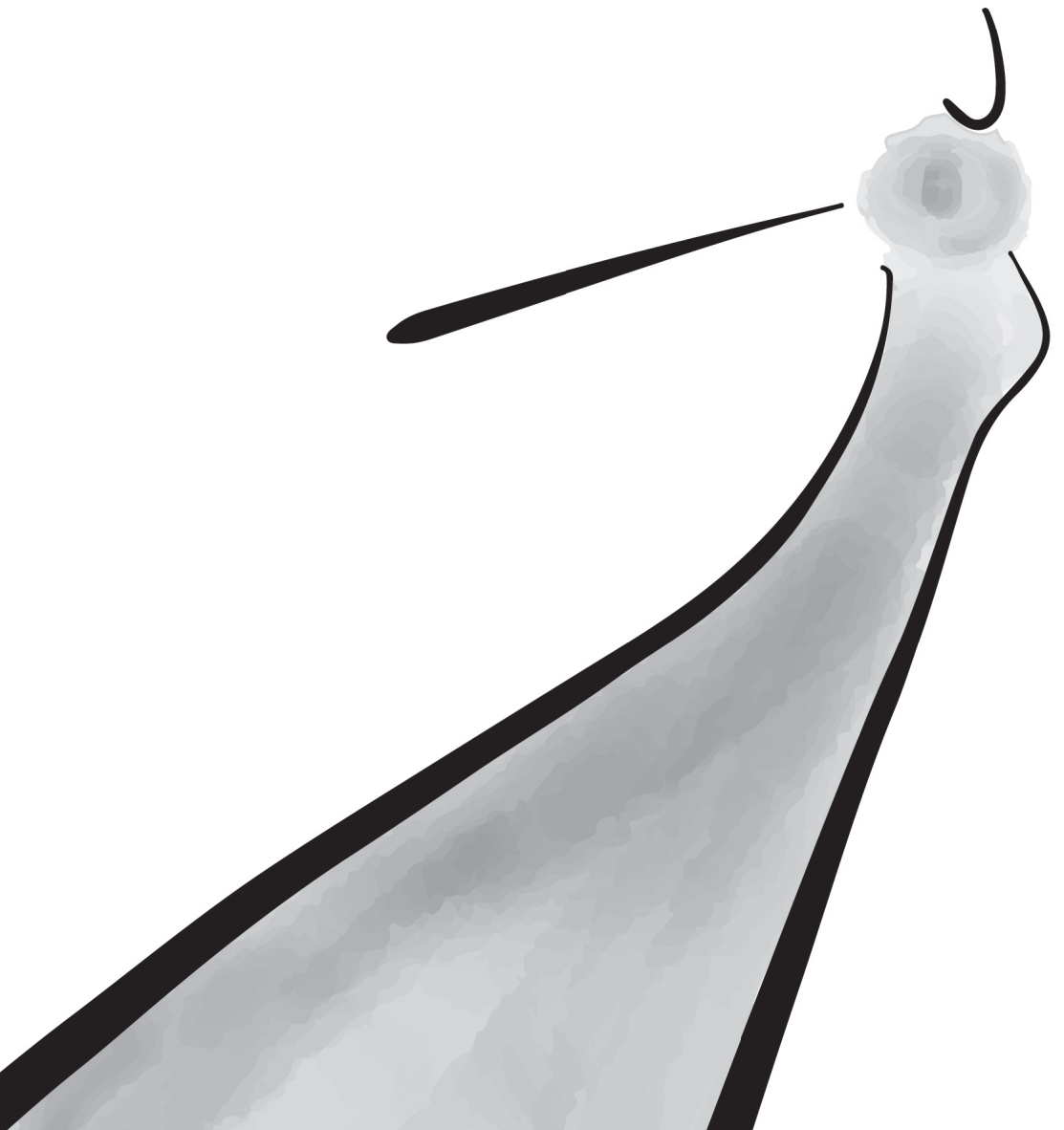

CHAPTER 2

Selenium status is positively associated with bone mineral density in healthy aging European men

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ABSTRACT

Objective

It is still a matter of debate if subtle changes in selenium (Se) status affect thyroid function tests (TFTs) and bone mineral density (BMD). This is particularly relevant for the elderly, whose nutritional status is more vulnerable.

Design and methods

We investigated Se status in a cohort of 387 healthy elderly men (median age 77 yrs; inter quartile range 75-80 yrs) in relation to TFTs and BMD. Se status was determined by measuring both plasma selenoprotein P (SePP) and Se.

Results

The overall Se status in our population was low normal with only 0.5% (2/387) of subjects meeting the criteria for Se deficiency. SePP and Se levels were not associated with thyroid stimulating hormone (TSH), free thyroxine (FT4), thyroxine (T4), triiodothyronine (T3) or reverse triiodothyronine (rT3) levels. The T3/T4 and T3/rT3 ratios, reflecting peripheral metabolism of thyroid hormone, were not associated with Se status either.

SePP and Se were positively associated with total BMD and femoral trochanter BMD. Se, but not SePP, was positively associated with femoral neck and ward's BMD. Multivariate linear analyses showed that these associations remain statistically significant in a model including TSH, FT4, body mass index, physical performance score, age, smoking, diabetes mellitus and number of medication use.

Conclusion

Our study demonstrates that Se status, within the normal European marginally supplied range, is positively associated with BMD in healthy aging men, independent of thyroid function. Thyroid function tests appear unaffected by Se status in this population.

Abbreviations

B, Beta; BMD, bone mineral density; BMI, body mass index; DM, Diabetes Mellitus; FT4, free thyroxine; IQR, inter quartile range; N, number of subjects; * , $p<0.05$; ** , $p<0.01$; ***, $p<0.001$; rT3, reverse triiodothyronine; SD, standard deviation, Se, selenium; (SE), standard error; SePP, selenoprotein P; TFTs, thyroid function tests; TH, thyroid hormone; TSH, thyroid stimulating hormone; T3, triiodothyronine; T4, thyroxine

INTRODUCTION

Selenium (Se) is a nutritional trace element that is essential for the biosynthesis of selenoproteins. Selenoproteins elicit important functions in different processes such as thyroid hormone (TH) homeostasis and antioxidant defence (1, 2). Patients with severely compromised selenoprotein biosynthesis or massive Se deficiency show a variety of endocrine defects including abnormal thyroid function tests (TFTs) and delayed or impaired bone formation (3-5). However, it is still a matter of debate if also more subtle changes in Se status are associated with alterations in TFTs and bone mineral density (BMD). This is especially important for elderly who are at risk for malnutrition (6).

Peripheral metabolism of TH levels is predominantly mediated by the selenoenzymes type 1, type 2, and type 3 deiodinase (D1-3), which all contain a selenocysteine in their catalytic centre. An altered metabolism of TH due to low Se status has been proposed as a mechanism for the age-dependent changes in thyroid parameters (1, 7-13).

In 2012 we described a positive association between Se status and BMD in postmenopausal women (9). No data on the association of Se status and BMD in elderly men are yet available (14-16), despite the knowledge that Se biology and selenoprotein expression show sex-specific differences in rodent models and human studies (17).

For those reasons we investigated the association between Se status, TFTs and BMD in a population of elderly men. Se status was determined by measuring both selenoprotein P (SePP) and plasma Se concentrations. SePP is a liver-derived Se storage and transport protein and is considered the most reliable biomarker of Se status (18).

MATERIALS AND METHODS

Study population

The Zoetermeer study is a cohort study conducted in clinically healthy independently living Caucasian elderly men between 1996 and 2000. The specific design and the effect of thyroid hormone concentrations on disease, physical function and mortality has been reported in 2005 (12). In brief, individuals were drawn from the municipal register of Zoetermeer, The Netherlands. Inclusion criteria were male sex, age at least 70 years, and a sufficient physical and mental status to visit the study center independently. The Medical Ethics Committee of the Erasmus Medical Center approved the

study, which included permission for additional measurements in stored serum and plasma samples. Four hundred and three men participated and gave written informed consent.
The subjects were interviewed by the same person and medical history, smoking status and medication use were recorded. A total of 16 individuals were excluded; 6 individuals on TH replacement, 8 individuals taking the thyroid hormone interfering drug amiodarone, and 2 Se outliers (defined as SePP or Se ≥4 standard deviations from the mean). This resulted in a final population of 387 subjects for analysis.

Determination of Se status

SePP and Se levels were measured in 2007 in the same plasma samples in parallel and blinded to the characteristics of the participants in a laboratory remote from the study site. A previous stability analysis showed no decline over time (19). Fluorescence spectroscopy was used for Se determination as described earlier (18). A commercial human serum standard (Sero AS, Billingstad, Norway) was included for standardization. SePP concentrations were determined by a luminometric immune assay as described (19). The analyses were conducted in duplicates and inter- and intra-assay variations were <15% during the measurements.
Normal values for SePP and Se were determined in our previous study including 2374 European postmenopausal woman, using the same spectroscopy and immune assay (9).
Reference ranges from another Dutch study in 1987 are not comparable due to the use of another Se assay (20). There is no association between Se status and sex, but there is a positive association with age (21). Therefore, all analyses are adjusted for age.

Thyroid function tests

Blood samples were collected in the morning after an overnight fast. Serum was separated by centrifugation and stored at −40 °C. All serum TFTs (thyroid stimulating hormone (TSH), free thyroxine (FT4), thyroxine (T4), triiodothyronine (T3) and reverse triiodothyronine (rT3) were determined using well-established assays as described previously (22).

Bone Mineral Density

Total BMD was measured using dual-energy x-ray absorptiometry (DEXA) (Lunar, Madison, WI), as were hip BMDs at the femoral neck, trochanter,

and Ward's triangle. Quality assurance for DEXA, including calibration, was performed every morning, using the standards provided by the manufacturer (12).

Physical performance, body composition and diabetes mellitus

Physical performance was assessed as described by Guralnik *et al.* (23), including measurements of standing balance, walking speed and ability to rise from a chair. Scores of the tests as well as the summary performance scale were comparable with subjects of the same age group investigated by Guralnik *et al.* (12, 23).

Height and weight were measured in standing position without shoes. Body mass index (BMI) was calculated as the weight in kilograms divided by the square of the height in meters. The average of two readings was used in the analyses.

Hemoglobin A1c (HbA1c) and fasting glucose levels were determined (24). Diagnosis of new onset diabetes mellitus (DM) was based on a fasting plasma glucose level ≥7.0 mmol/L and HbA1c ≥6.5 percent (25).

Statistics

Analyses were performed using SPSS version 23 (SPSS Inc, Chicago, Il). Normal distribution was evaluated using the Kolmogorov-Smirnov test, and variables that were not normally distributed underwent natural logarithmic transformation. Linear regression analyses were used to determine the associations between SePP and Se, TFTs and BMD. ANOVA analysis was used to present mean values for the lowest and highest Se and SePP quartiles to provide extra insight into the actual effects.

Multivariate linear model was used to correct the association between Se status and BMD for TSH, FT4, BMI, physical performance score, smoking status, known and new onset DM and total number of medication use. All analyses were adjusted for age. A p-value <0.05 was considered significant.

RESULTS

Baseline characteristics and selenium status

The median age of the population was 77 yrs [inter quartile range (IQR) 75-80 yrs; range 73-94yrs]. The mean SePP concentration was 3.4 mg/L (SD±0.75) and median Se was 92 µg/L [IQR 82-101]. Overall Se status was suboptimal,

but only 0.5% (2/387) of subjects met the criteria for Se deficiency (SePP < 2 mg/L and Se < 58 µg/L) (9) (Table 1).
Thirty one subjects with known DM and 13 patients with new onset DM were identified. The prevalence of 11.4% is conform the large population based study in the Netherlands including 1614 patients aged >70 years (26), but lower compared to the age and sex specific prevalence of 16% from the European cohorts combined (27).

Table 1. Baseline characteristics

	Normal value	N=387
Age, yrs, median [IQR]		77[75-80]
SePP, mg/L, mean (±SD)	≥ 2.0	3.44(±0.75)
Se, µg/L, median [IQR]	≥ 58	91.9[82.0-101.1]
TSH, mU/L, median [IQR]	0.4-4.3	0.95[0.60-1.43]
FT4, pmol/L, mean (±SD)	11-25	16.6(±3.1)
Total BMD, mg/cm2, mean (±SD)		1169.1(±98.0)
Smoking, no (%)		66(17.1%)
Physical performance score, median [IQR]	0-12	9[7-10]
BMI, kg/m² , mean (±SD)	18.5-25.0	25.4(±3.0)
DM, no (%)		44(11.4%)
Medication, no, median [IQR]		1[0-2]

BMD, bone mineral density; BMI, body mass index; DM, diabetes mellitus pre-existent and new onset; FT4, free thyroxine; IQR, inter quartile range; medication, total number of medication, N, total number of subjects studied; no, number; yrs, years; SD, standard deviation; Se, Selenium; SePP, selenoprotein P; TSH, thyroid stimulating hormone

Thyroid function tests

SePP and Se levels were not associated with TFTs (Table 2). TH levels depend not only on the activities of TH-metabolizing enzymes but also, among other things, on thyroid function and plasma TH-binding capacity. Therefore, ratios between plasma TH's are thought to better reflect tissue deiodinase activities (28). However, T3/T4, T3/rT3 and rT3/T4 ratios were not associated with Se status either (Table 2).

Bone mineral density

SePP and Se were positively associated with total BMD and femoral trochanter BMD. Se, but not SePP, was positively associated with femoral neck BMD and Ward's triangle BMD (Table 3). We subsequently constructed a multivariate linear regression model to control for a number of potentially interfering

Table 2. Associations between selenium status and thyroid function tests

		SePP				Se			
		Linear regression		Quartiles		Linear regression		Quartiles	
TFTs	Normal value	*B(SE)*	*p*	Q1 Mean(SE)	Q4 Mean(SE)	*B(SE)*	*p*	Q1 Mean(SE)	Q4 Mean(SE)
TSH, mU/L	0.4-4.3	<0.01(0.07)	0.95	1.24(0.10)	1.14(0.10)	<0.01(0.03)	0.98	1.23(0.10)	1.30(0.10)
FT4, pmol/L	11-25	0.11(0.21)	0.60	16.4(0.33)	16.7(0.32)	0.01(0.01)	0.11	16.2(0.32)	16.7(0.32)
T4, nmol/L	58-128	1.73(1.07)	0.11	79.1(1.68)	81.3(1.64)	0.03(0.05)	0.45	80.1(1.66)	79.2(1.65)
T3, nmol/L	1.43-2.51	<0.01(0.02)	0.92	1.45(0.03)	1.41(0.02)	<0.01(0.01)	0.80	1.40(0.02)	1.41(0.02)
rT3, nmol/L	0.14-0.34	0.01(0.01)	0.22	0.32(0.01)	0.33(0.01)	<0.01(0.01)	0.96	0.33(0.01)	0.32(0.01)
T3/T4 x100	1.42-3.05	-0.05(0.03)	0.11	1.95(0.05)	1.78(0.05)	<0.01(0.01)	0.95	1.82(0.05)	1.89(0.05)
T3/rT3	3.12-13.03	-0.15(0.11)	0.30	5.01(0.16)	4.56(0.16)	<0.01(0.01)	0.95	4.69(0.16)	4.72(0.16)
rT3/T4x100	0.15-0.44	<0.01(0.01)	0.97	0.41(0.01)	0.41(0.01)	<0.01(0.01)	0.84	0.42(0.01)	0.42(0.01)

The results (*B(SE)* and corresponding *p*-values) of the linear regression analyses of SePP and Se levels versus various TFTs are shown. In addition, mean values for the lowest and highest Se and SePP quartiles are presented to provide extra insight into the actual effects.

B, Beta; FT4, free thyroxine; rT3, reverse triiodothyronine; Se, Selenium; (SE), standard error; SePP, selenoprotein P; TFTs, thyroid function tests; TSH, thyroid stimulating hormone; T3, triiodothyronine; T4, thyroxine; Q1, first quartile; Q4, fourth quartile

Table 3. Selenium status and association with bone mineral density

BMD mg/cm²	SePP					
	Linear regression		Multivariate regression		Quartiles	
	B(SE) *Unadjusted*	*p Unadjusted*	*B*(SE) *Adjusted*	*p Adjusted*	Q1 Mean(SE)	Q4 Mean(SE)
Total	15.57(6.68)	0.02*	13.36 (6.09)	0.03*	1147.6(10.1)	1187.6(9.9)
Femoral neck	13.42(10.15)	0.14	10.72(9.91)	0.21	855.5(15.3)	899.9(15.1)
Femoral trochanter	26.35(10.23)	0.01*	23.80(9.52)	0.01*	812.7(15.4)	881.7(15.2)
Femoral ward	16.22(11.30)	0.08	12.55(11.14)	0.15	688.6(17.1)	736.4(16.8)

BMD mg/cm²	Se					
	Linear regression		Multivariate regression		Quartiles	
	B(SE) *Unadjusted*	*p Unadjusted*	*B*(SE) *Adjusted*	*p Adjusted*	Q1 Mean(SE)	Q4 Mean(SE)
Total	0.87(0.28)	0.001**	0.81(0.26)	0.001**	1155.6(9.9)	1195.6(9.8)
Femoral neck	1.36(0.42)	0.001**	1.24(0.41)	0.002**	848.6(15.0)	924.5(14.7)
Femoral trochanter	1.50(0.43)	<0.001***	1.40(0.40)	<0.001***	813.9(15.0)	903.6(14.8)
Femoral ward	1.38(0.47)	0.003**	1.25(0.47)	0.006**	678.4(16.7)	760.3(16.5)

The results (*B*(SE) and corresponding *p*-values) of the linear regression analyses of SePP and Se levels versus BMD are shown. Multivariate linear model was used to correct for TSH, FT4, age, BMI, physical performance score, smoking status, known and new onset diabetes mellitus and total number of medication use (*B*(SE) adjusted and corresponding adjusted *p*-values).

Mean values for the lowest and highest Se and SePP quartiles are presented to provide extra insight into the actual effects.

B, Beta; BMD, bone mineral density; BMI, body mass index; FT4, free thyroxine; Se, Selenium; (SE), standard error; SePP, selenoprotein P; TSH, thyroid stimulating hormone; Q1, first quartile; Q4, fourth quartile; * , $p<0.05$; ** , $p<0.01$; ***, $p<0.001$

factors including; TSH and FT4, as measures of (mild) thyroid dysfunction, BMI, which is known to be a risk factor for osteoporosis and associated with food intake and smoking which is described to modify the antioxidant effect of Se on BMD (14). The association of DM, Se status and BMD is still a matter of debate (29). Therefore subjects with known and new onset DM are included in the model.

To account for the influence of chronic diseases and physical activity we also included the total number of medication use and physical performance score in the model.

After additional adjustment for these factors the positive associations between Se status and BMD remained statistically significant (Table 3).

DISCUSSION

In the present study we investigated Se status in healthy elderly men in relation to TFTs and BMD. Although elderly subjects are at increased risk of nutritional deficiencies, with our assay that is well suited to cover low SePP concentration ranges, only a few patients were Se deficient. This is an important finding, especially since Se deficiency is becoming increasingly recognized as a health risk. These results are in agreement with the vast majority of studies that all conclude that European subjects are marginally supplied and on average below the Se concentration needed for full expression of selenoproteins (30, 31). This is the first study to show in men that Se status, even within this low normal range, is positively associated with BMD independent of TH status. This is in concordance with our recent findings in elderly postmenopausal women (9).

Although an altered metabolism of TH due to low Se status has been proposed as a mechanism for the age-dependent changes in TFTs (7, 8), extensive profiling of thyroid parameters in the current study did not reveal any association of TFTs with Se status. Our analysis included assessment of T3/T4 and T3/rT3 ratios as a reflection of the peripheral metabolism of TH. We can therefore conclude that our previously reported association between FT4 and rT3 levels with physical performance and/or survival in this cohort is not mediated via Se status (12). The lack of association between Se status and TFTs in the current study is in line with a randomized controlled trial in elderly in which Se supplements failed to improve thyroid function (10). In addition, also in Se-deficient transgenic mice, the synthesis and metabolism of TH is surprisingly well maintained (32, 33).

These results are in contrast, however, with our recent study in 1144 healthy euthyroid postmenopausal women in which Se and SePP were inversely associated with FT3 and FT4 and positively associated with the T4/T3 ratio (9). The Se status of these two populations is comparable. This may point towards a sex-specific difference in this interaction, in line with a number of other sexual dimorphisms in Se metabolism in patients as well as in experimental animals (17). Notably, expression of D1 strongly differed between male and female rodents via Se-dependent mechanisms affecting protein translation (34). Unfortunately, no serum rT3 levels were available in the previous study on postmenopausal females, which would have allowed for a better comparison and speculation on the differential effects of Se status and peripheral deiodinase activities between elderly males and females. Discrepancies between the two studies might also be explained by the relatively older age of the current male population (77.8 (±3.6) versus 67.8 (±7.0) yrs). Older subjects may have more co-morbidities which also affect the degradation of TH.

Low Se status is known to be associated with skeletal disease in patients with mutations in selenoproteins (selenocystein insertion sequence binding protein 2), Kashin-Beck osteoarthropathy and women (4, 9). Also, Se intake appears to be inversely associated with the risk of osteoporotic hip fractures (14). Women are known to be more vulnerable to osteoporosis (35), but our current findings demonstrate that Se status influences BMD in men as well. Although only two individuals had subnormal Se values, it is very interesting that even in a population with borderline sufficiency there is a significant association with bone mineral density. An effect of TH on BMD could be excluded as correction for thyroid status did not affect the observed associations between Se status and BMD. Some previous clinical studies in healthy women did not demonstrate an association between Se status and BMD, possibly due to a lack of power (77 and 107 subjects) (15, 36). Mechanistically, SePP has been shown to transport Se to bone, and a receptor-mediated uptake ensures a relatively high bone Se supply even during Se shortage (37). *In vitro* studies have demonstrated an effect of Se on osteoblast differentiation and subsequent bone resorption by modulating oxidative stress (38, 39).

Our study has a number of potential limitations. Due to the cross-sectional design of the study, causality of the associations found cannot be assessed. We cannot exclude that the voluntary participation of our subjects has resulted in a bias with more health-interested and thus better eating elderly

being enrolled. In addition, no data on vitamin D or calcium levels were available. In our previous paper the association of Se status and BMD was not influenced by vitamin D (9). To the best of our knowledge there is no clear evidence that plasma calcium levels are associated with Se status., Parathyroid hormone which reflects changes in calcium homeostasis, did not influence the association of Se status and BMD in previous studies either (9). Therefore, it is not very likely that in the current study in healthy ambulant men, differences in vitamin D or calcium levels have confounded or mediated our results. Finally, while we have shown effects on BMD, no data on fracture risk were available. Future studies should therefore investigate the relation between Se levels and fracture risk, as well as the underlying pathophysiological mechanisms of these observed associations.

CONCLUSIONS

Our study demonstrates that Se status within the low normal range is positively associated with BMD in healthy aging European men, independent of TH function. TFTs appear unaffected by Se status in this population.

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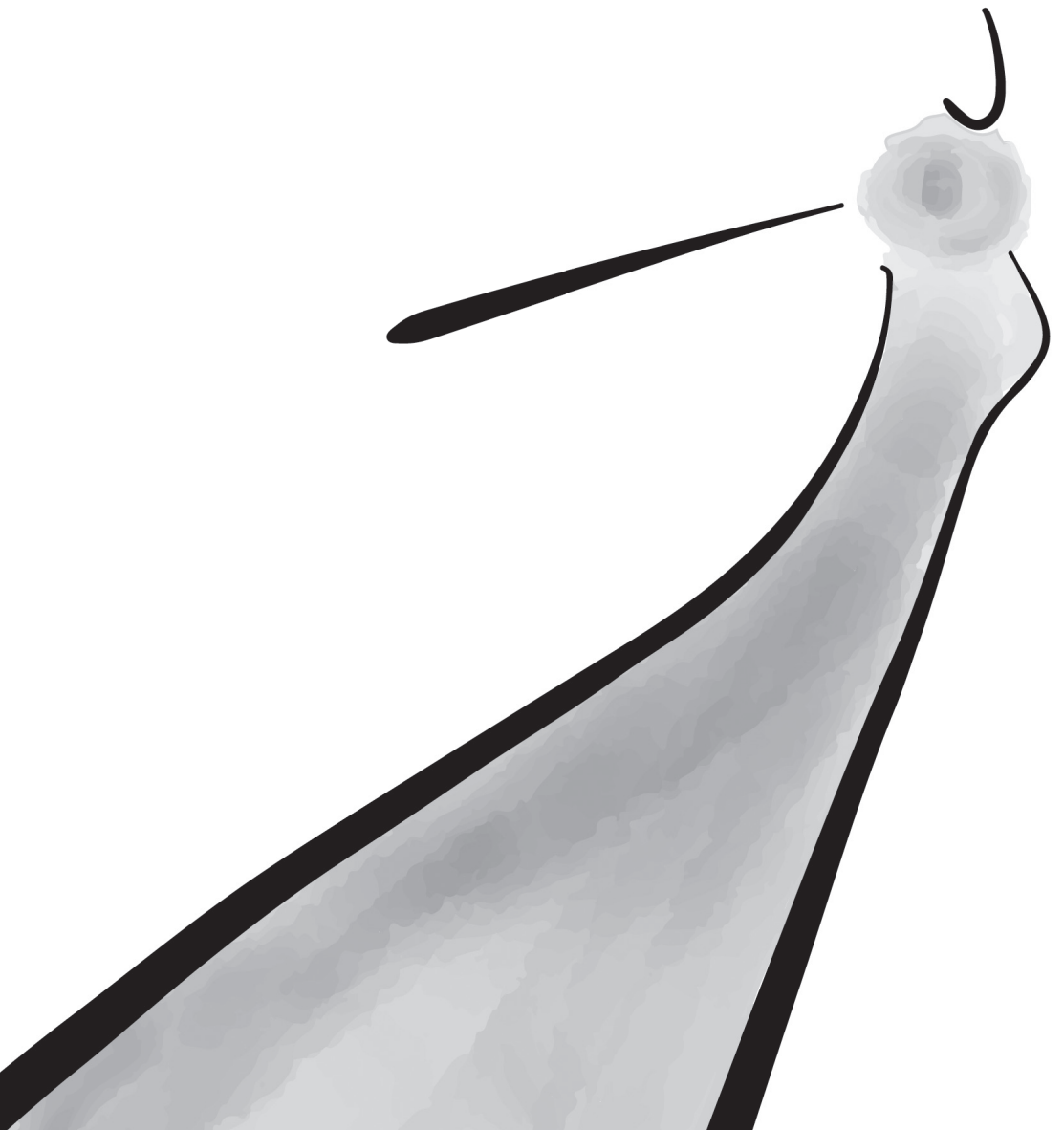

CHAPTER 3

Sorafenib-induced changes in thyroid hormone levels in patients treated for hepatocellular carcinoma

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ABSTRACT

Context
The pathogenesis of tyrosine kinase inhibitor-induced thyroid hormone (TH) alterations are still a matter of debate.

Objective
The objective of this study was to determine the effects of sorafenib on TH levels in patients with hepatocellular carcinoma (HCC) and to evaluate possible mechanisms.

Design
We performed a prospective cohort study between 2009 and 2016.

Setting
This study was conducted at a tertiary referral center.

Patients
This study included 57 consecutive patients with HCC who were treated with sorafenib.

Main Outcome Measure
Thyroid-stimulating hormone (TSH) and free thyroxine (FT4) levels were measured every 6 weeks, and extensive thyroid function tests (TFTs) were measured before treatment (t0), after 6 weeks (t6), and at the end of therapy. The effect of sorafenib on TH transport by monocarboxylate transporter (MCT)8 or MCT10 was tested in transfected COS1 cells.

Results
Four patients (7%) developed thyroiditis. Among the other patients, 30% had elevation of TSH or FT4 above the normal range. Overall, between t0 and t6, mean TSH increased from 1.28 to 1.57 mU/L ($p < 0.001$) and mean FT4 from 18.4 to 21.2 pmol/L ($p < 0.001$). Simultaneously, the serum triiodothyronine (T3)/reverse triiodothyronine ratio and the (T3/thyroxine) ×100 ratio decreased. Sorafenib decreased cellular T3 uptake by MCT8 and to a lesser extent by MCT10.

Conclusions

These clinical data suggest that sorafenib affects TFTs on multiple levels. Our *in vitro* experiments suggest a possible role of sorafenib-induced inhibition of T3 transport into the cell by MCT8 and MCT10.

Abbreviations

CI, confidence interval; D3, deiodinase type 3; FT4, free thyroxine; HCC, hepatocellular carcinoma; HPT, hypothalamic-pituitary-thyroid; IQR, interquartile range; MCT, monocarboxylate transporter; NTI, nonthyroidal illness; PFS, progression-free survival; rT3, reverse triiodothyronine; t0, before treatment; t6, after 6 weeks of treatment; T3, triiodothyronine; T4, thyroxine; TH, thyroid hormone; TFT, thyroid function test; TKI, tyrosine kinase inhibitor; TPO-Ab, thyroid peroxidase antibody; TSH, thyroid-stimulating hormone; TSHR-Ab, thyroid-stimulating hormone receptor antibody; WHO, World Health Organization

INTRODUCTION

Several tyrosine kinase inhibitors (TKIs) are reported to be associated with changes in thyroid function tests (TFTs), of which sunitinib is the best studied (1). Sorafenib is used in the treatment of advanced hepatocellular carcinoma (HCC), renal cell carcinoma, and radioactive iodine-resistant differentiated thyroid carcinoma (DTC) (2-4). It has antiangiogenic, antiproliferative, and proapoptotic effects via multiple effector mechanisms, such as inhibition of the vascular endothelial growth factor receptor (5). In 2011, we reported on two patients with sorafenib-induced thyroiditis (6); since then, other cases of sorafenib-induced thyroiditis have been described (7). Hypothyroidism and adverse effects, such as hypertension, are described to be associated with better prognosis among patients undergoing treatment with TKIs (8, 9). The incidence of sorafenib-induced hypothyroidism varies from 18% to 50%. In previous studies (10-15), the diagnosis of sorafenib-induced hypothyroidism was only based on increased thyroid-stimulating hormone (TSH) levels. Patients with preceding thyroiditis, a different entity contributing to TSH increase, were not excluded. The pathogenesis of the rise in TSH in nonthyroiditis cases has not been elucidated.

A study in athyroid patients treated with sorafenib suggests that there may be an enhanced peripheral degradation of thyroid hormone (TH) by deiodinase type 3 (D3) (16). D3 expression has been described in other tumors (17). Inhibition of TH uptake via the cellular TH transporter [monocarboxylate transporter (MCT) 8] has been shown for sunitinib, imatinib, dasatinib, and bosutinib, but this has not been studied for sorafenib (18). The effects of TKIs on MCT10-mediated TH uptake into cells are, to the best of our knowledge, unknown.

The aim of the present study was to explore the effects of sorafenib on the hypothalamic-pituitary-thyroid (HPT) axis and peripheral TH metabolism by assessing detailed TFTs in patients without known thyroid disease. By studying patients with intact thyroid glands, we were able to study effects on the HPT axis at different levels. In addition, we studied the consequences of sorafenib on cellular triiodothyronine (T3) uptake *in vitro*.

PATIENTS AND METHODS

Patient characteristics

We prospectively studied consecutive patients with progressive metastatic HCC and Child-Pugh A status who were treated with sorafenib between

January 2007 and February 2016 in the Erasmus MC Cancer Institute, Rotterdam, Netherlands. Patients routinely started sorafenib at a dose of 400 mg, which was increased in 1 month to 800 mg if deemed safe by the treating physician.

Routine TFT [TSH and free thyroxine (FT4)] levels were determined every 6 weeks. In patients with enough material available, more extensive TFT levels were measured at baseline (t0), after 6 weeks (t6), and at the end of sorafenib therapy (Supplemental Figure 1). Patients with at least two laboratory evaluations were included in the study. The study was reviewed by the medical ethical committee of the Erasmus Medical Center (MEC-2015-755); requirements to obtain informed consent were waved.

Clinical outcome

Baseline World Health Organization (WHO) performance status was assessed by the treating physician. Progression-free survival (PFS) was computed as the time from treatment initiation to disease progression according to the treating physician or death (19). Overall survival was computed as the time from treatment initiation to death. Adverse events were scored following Common Terminology Criteria for Adverse Events v4.0 (20). The incidence of all-grade hand foot skin reaction, hypertension, gastrointestinal complaints and thrombocytopenia was scored. Severe liver toxicity criteria were grade 3 or 4 liver test disturbances in liver transaminases, γ-glutamyl transferase, and alkaline phosphatase or grade 2 bilirubin disturbances.

TFTs

Serum was centrifuged and stored at −20°C immediately after withdrawal. TSH (reference value, 0.4 to 4.3 mU/L) was measured using the Immulite 2000 platform (Siemens, Erlangen, Germany). FT4 (reference value, 11 to 25 pmol/L), thyroxine (T4) (reference value, 58 to 128 nmol/L), and T3 (reference value, 1.4 to 2.5 nmol/L) were measured using the Vitros ECi immunoanalyzer (Ortho-Clinical Diagnostics, Raritan, NJ). Reverse triiodothyronine (rT3) (reference value, 0.21 to 0.54 nmol/L) was measured by in-house radioimmunoassay (21). Intra- and interassay variability coefficients of all assays were <11% (22). T3/rT3 (reference value, 2.65 to 7.65) and rT3/T4 ×100 (reference value, 1.4 to 3.1) ratios were calculated as a proxy for peripheral deiodinase activity. Thyroid peroxidase antibodies (TPO-Abs) (reference value, <100 IU/mL) were measured using the ImmunoCAP method (Phadia 250, Uppsala, Sweden). TSH receptor antibodies (TSHR-

Abs) (reference value, <0.9 IU/L) were measured using the 2009-2012 TRAK LIA test (Brahms, Hennigsdorf, Germany) and the 2012-2015 TRAK Kryptor test (Brahms, Hennigsdorf, Germany), both WHO calibrated.

In vitro experiments

Effects of sorafenib on cellular uptake of T3 by human MCT8 and MCT10

Materials

Dulbecco's phosphate-buffered saline with calcium and magnesium and GlutaMAX medium was obtained from Life Technologies (Bleiswijk, the Netherlands); culture dishes were from Corning (Schiphol, Netherlands); COS1 cells were from ATCC; bovine serum albumin, d-glucose, and T3 were from Sigma-Aldrich (Zwijndrecht, Netherlands); transfection reagent X-tremeGENE9 were from Roche (Almere, Netherlands); and $Na^{125}I$ was from Perkin-Elmer (Groningen, Netherlands). [^{125}I] T3 was prepared in our laboratory as described previously (22). The human MCT8 plasmid pcDNA3-hMCT8 and the human pcDNA3-hMCT10 were obtained as described elsewhere (22).

Cellular T3 uptake assays

COS1 cells were cultured in 24-well dishes with 0.5 mL Dulbecco's modified Eagle medium/F12 + GlutaMAX medium containing 9% heat-inactivated fetal bovine serum, 2% penicillin/streptomycin, and 100 nM Na_2SeO_3. The cells were transfected with 100 ng empty pcDNA3, pcDNA3-hs MCT8, or pcDNA3-MCT10 as described (23). T3 uptake was tested 48 hours after transfection. The cells were washed with the assay buffer (Dulbecco's phosphate-buffered saline $CaCl_2$ + $MgCl_2$ + 0.1% bovine serum albumin + 0.1% glucose) and incubated for 5 minutes at 37°C with 1 nM (10^5 cpm) [^{125}I] T3 and 0, 1, 10, or 100 μM sorafenib or 0, 1, 10, or 100 μM sunitinib in 0.5 mL assay buffer. After incubation, cells were washed with the assay buffer, lysed with 0.1 M NaOH, and counted in a gamma counter. Data were obtained from three independent experiments, each performed in duplicate.

D3 activity in HCC samples

To exclude a major contribution of D3 expression in HCC to the altered TFTs, we measured D3 in six random patients from whom presorafenib HCC biopsies were available. Tumor tissue was fresh frozen and stored at −80°C until use. Thawed tissue samples were homogenized on ice in 10 volumes of PED10 buffer [0.05 M phosphate, 1 mM EDTA (pH 7.2), 10 mM DTT] using

a Polytron (Kinematica AG, Lucerne, Switzerland). Liver D3 activities were measured in duplicate by incubation of tissue homogenate (250 µg protein) for 120 minutes at 37°C with 1 nM [3′-^{125}I]T3 (200,000 cpm) in 0.1 mL PED10 as described elsewhere (24).

Statistical analysis

Normal distribution was evaluated using the Kolmogorov-Smirnov test. Residuals that were not normally distributed underwent natural logarithmic transformation, and if still skewed *p*-values were obtained via bootstrapping. Changes in TFTs were analyzed using a paired sample *t* test and if not normally distributed via a paired Wilcoxon signed-rank test. False discovery rate correction for multiple comparisons proposed by Benjamini (25) was applied. Logistic regression analysis was used to determine the association between change in TFTs and adverse effects. Median survival time was calculated using Kaplan Meier. A Cox proportional-hazard regression model adjusted for age, sex, WHO performance status, and average dose of sorafenib was used for the survival analysis. Proportional hazard and linearity assumptions were met. Two-way ANOVA with correction for repeated testing and *post hoc* analysis with a paired *t* test was used for sorafenib-induced inhibition of MCT8 and MCT10 transport of T3 into cells. Analyses were performed using SPSS version 23 (SPSS Inc., Chicago, IL).

RESULTS

Patient characteristics

Blood samples were collected from 57 patients with HCC. One patient had a TSH ≥10 mU/L before therapy and was therefore excluded from the analyses. None of the patients had pre-existent hyperthyroidism or hypothyroidism or used drugs interacting with TFTs, such as amiodarone and corticosteroids. This resulted in a final population of 56 patients, 44 (79%) of whom were male. Median age was 67 years [interquartile range (IQR), 57 to 71 years], and median WHO performance status was 1 (IQR, 1 to 2).

Thyroiditis

Four patients (7%) developed thyroid disease, with a pattern consistent with thyroiditis. Two of these patients have been reported in detail (6), with clearly elevated TPO-Ab (866 IU/mL) or TSHR-Ab (368 IU/L) levels at the time of thyroiditis. The other two patients also showed markedly increased TPO-Ab (1302 and 439 IU/mL) and TSHR-Ab (19 IU/L) levels at the time of

thyroiditis. Prospectively, both patients had elevated TPO-Ab levels before initiation of sorafenib treatment (140 and 343 IU/mL). In comparison, none of the 52 patients without thyroiditis had TPO-Abs, and only six patients (12%) showed mildly elevated TSHR-Ab levels (median, 1.7 IU/L; IQR, 1.1 to 1.9). Ultrasound was not routinely performed prospectively.
Patients with thyroiditis had a median PFS of 16.3 months [95% confidence interval (CI), 6.1 to 26.5], and patients without thyroiditis had a median PFS of 4.9 months (95% CI, 2.3 to 7.5). Median overall survival was 18.5 months (95% CI, 0.1 to 43.5) vs. 10.8 months (95% CI, 8.6 to 13.0), respectively. We refrained from statistical analysis due to small patient number.

TFTs

Patients with thyroiditis were excluded from subsequent analyses of TFTs. Five out of the remaining 52 patients had mild subclinical baseline thyroid dysfunction: three patients had an isolated TSH elevation (5.14, 5.15, and 6.59 mU/L), and two patients had a low TSH (0.25 and 0.39 mU/L).
In 14 of the other 47 patients (30%), TSH or FT4 became elevated above the upper limit of normal during treatment. Overall, TSH and FT4 levels rose significantly after start of treatment (Figure 1). Similarly, rT3 and T4 levels increased significantly (Table 1), whereas the serum T3/rT3 and T3/T4 ratios significantly decreased (Figure 1). These changes in TFTs occurred within 6 weeks after start of treatment and persisted until the end of treatment.

Table 1. Change in TFTs

	Reference values	***t0*** *median [IQR]*	***t6*** *median [IQR]*	***p*** ***t0 vs. t6***	**tω** *median [IQR]*	***p*** ***t0 vs. tω***
Parameter						
TSH(mU/L)	0.4-4.3	1.28[0.88- 1.66]	1.57[1.23-2.78]	<0.001***	1.68[1.03-2.65]	<0.001***
FT4(pmol/L)	11-25	18.4[16.9-19.9]	21.2[18.5-24.8]	<0.001***	22.1[18.4-26.6]	<0.001***
T4(nmol/L)	58-128	115[101-138]	121[98-152]	0.01*	123[108-144]	0.02*
T3(nmol/L)	1.43-2.51	2.12[1.87-2.39]	2.06[1.73-2.31]	0.12	1.95[1.75-2.17]	0.001**
rT3(nmol/L)	0.21-0.54	0.41[0.36-0.59]	0.57[0.41-0.73]	<0.001***	0.58[0.46-0.76]	<0.001***
Ratio						
T3/rT3	2.65-7.65	4.84[3.74-5.84]	3.68[2.63-5.01]	<0.001***	3.40[2.73-3.99]	<0.001***
T3/T4x100	1.42-3.05	1.88[1.56-2.05]	1.58[1.41-2.03]	0.003**	1.55[1.29-1.81]	<0.001***

Changes in TFT were analyzed using a paired sample t test and if not normally distributed via a paired Wilcoxon signed-rank test. False discovery rate correction for multiple comparisons proposed by Benjamini (25) was applied. IQR, interquartile range; *, p<0.05; ** , p<0.01; ***, p<0.001

N, number of patients; t0, before treatment; t6, after 6 weeks of treatment; tω, at the end of therapy; TFT, thyroid function tests

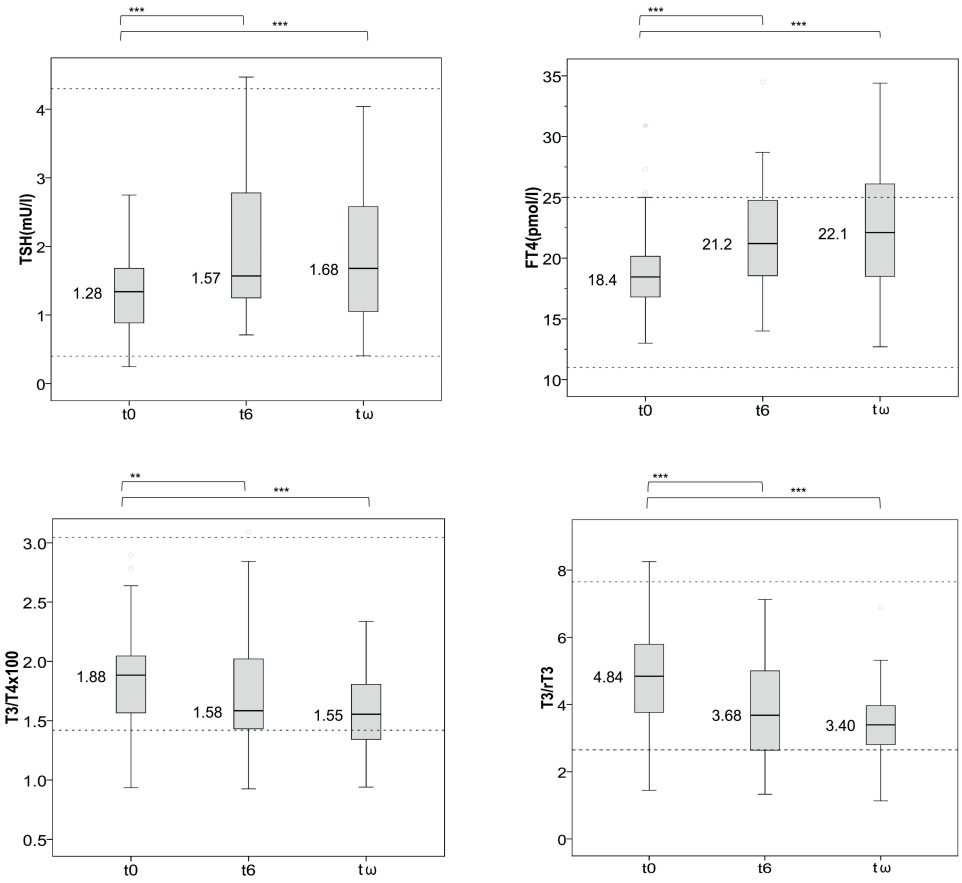


Figure 1. Change in TFTs at t0, t6, and end of therapy (tω). Changes in TFTs were analyzed using a paired sample t test and if not normally distributed via a paired Wilcoxon signed-rank test. False discovery rate correction for multiple comparisons proposed by Benjamini (25) was applied. **, $p < 0.01$; ***, $p < 0.001$.

In vitro experiments

Cellular T3 uptake mediated by MCT8 was significantly and dose-dependently inhibited by sorafenib, and very similar effects were observed with sunitinib (Figure 2). Both sorafenib and sunitinib had marginal effects on T3 uptake by control cells transfected with empty vector and on T3 uptake of cells transfected with MCT10. The short exposure of the cells to sorafenib and sunitinib (5 minutes) and the differential effects of the inhibitors on MCT8-mediated vs. MCT10-mediated and background T3 uptake argue against an important contribution of possible cytotoxic effects of sorafenib and sunitinib in these experiments.

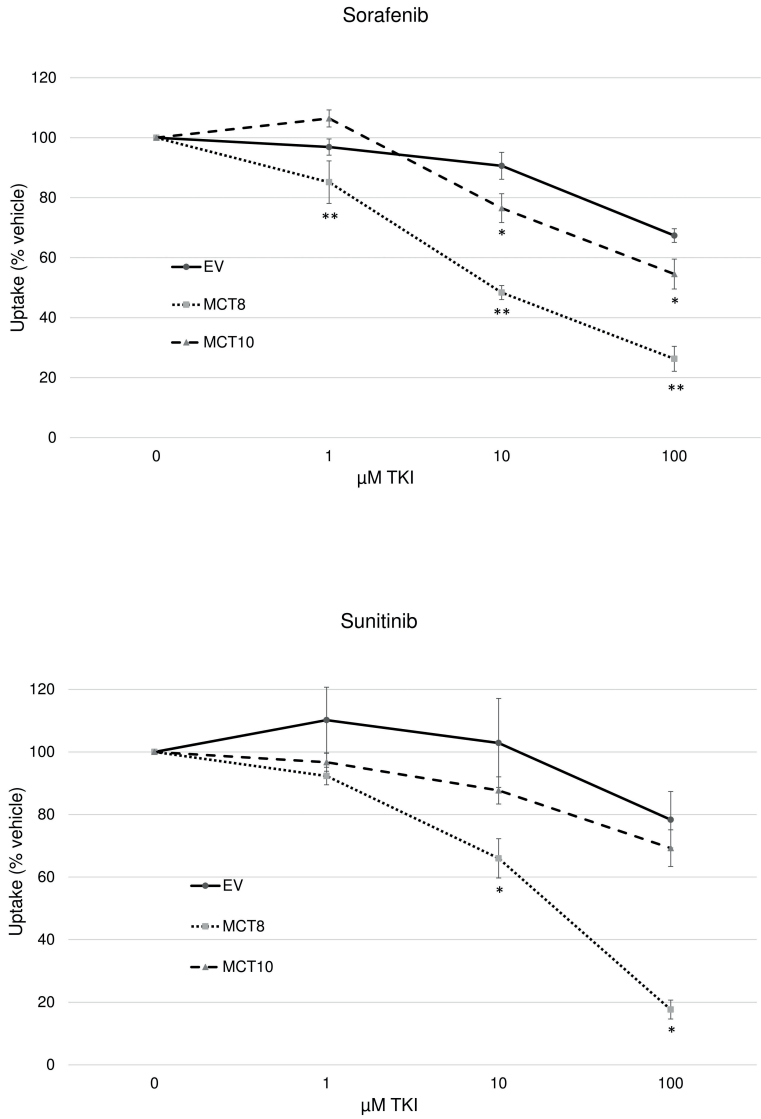


Figure 2. Effects of sorafenib and sunitinib on T3 uptake by MCT8 or MCT10. Cells were incubated for 5 minutes with 1 nM [^{125}I]T3 in the absence or presence of 1, 10, or 100 µM sorafenib or sunitinib as described in Materials and Methods. T3 uptake by cells transfected with MCT8 or MCT10 is corrected for T3 uptake by control cells transfected with empty vector (EV). T3 uptake is expressed as mean (standard error of mean) percentage of that in the absence of sorafenib or sunitinib of three independent experiments each performed in duplicate. Two-way ANOVA with correction for repeated testing showed significant change in T3 uptake after for sorafenib ($p < 0.02$) and sunitinib ($p < 0.05$). Post hoc, the significance of the difference between the effects of sorafenib or sunitinib on T3 uptake by MCT8 or MCT10 expressing cells vs. control cells is shown in the figure. This was tested by paired t test. *$p < 0.05$; **$p < 0.01$.

The average D3 activity in the HCC tissue before sorafenib treatment was 1.17 (IQR, <0.1 to 1.63) fmol/min/mg protein. This was not increased compared with the D3 activities in historical control livers (liver biopsies of patients who died of severe brain damage, taken within minutes after death) that we studied previously (26).

Clinical outcome

An increase in TSH level was associated with a deterioration of PFS (Supplemental Table 1). This negative effect of TSH on PFS persisted in a multiple Cox-regression analysis with correction for age, sex, WHO performance status, and average dose of sorafenib (Figure 3). Adding response evaluation criteria in solid tumors (27) to the model did not influence the results. There was no association of FT4 with PFS. TSH showed the same trend for overall survival but did not reach statistical significance (Supplemental Table 1). Changes in TFTs were not correlated with adverse events (Supplemental Table 1).

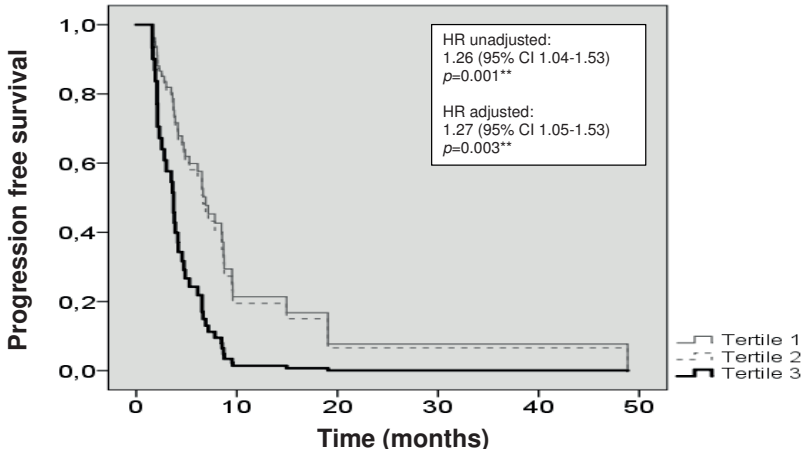


Figure 3. The proportion of progression free survival by ΔTSH tertiles. Adjusted hazard ratio (HR) was calculated in a Cox proportional-hazard regression model adjusted for age, sex, WHO performance status, and average dose of sorafenib.

DISCUSSION

In this cohort of sorafenib-treated patients with HCC, we demonstrate that sorafenib affects TFTs via multiple mechanisms. Thyroiditis occurred in a small percentage of patients, whereas there was a combined increase in TSH

and FT4 in the remaining patients, which suggests a central effect on the HPT axis. Finally, there was a decrease in the T3/rT3 and T3/T4 ratios, suggesting additional effects on the peripheral metabolism of TH.

Thyroiditis

The incidence of thyroiditis was 7%, compared with 23% (14) and 5% (11) in other sorafenib cohorts and 40% (28) in patients treated with sunitinib. The incidence of subacute thyroiditis in the general population is much lower at around 12.1 cases per 100.000/y (29). However, we cannot draw conclusions on the basis of our observations because only four patients developed thyroiditis in this cohort.

Altered sensitivity of the HPT axis

Thirty percent of patients developed TSH or FT4 levels above the normal range during treatment. Median TSH and FT4 levels rose significantly. This simultaneous increase of TSH and FT4 suggests an altered setpoint of the HPT axis because an increase in FT4 would normally be accompanied by a decrease in TSH. For other TKIs, such as axitinib, an isolated increase in TSH accompanied by TH levels within the normal range has also been described (30). This altered setpoint could be explained at several levels because not only serum levels of FT3 and FT4 but also TH transport into the cell and intracellular deiodinase activity are determinants of TH action and the HPT axis setpoint (31).

Our *in vitro* experiments showed inhibition of MCT8-mediated T3 uptake by sorafenib. MCT8 is one of the transporters that is highly expressed in brain and pituitary (32), suggesting that sorafenib-induced interference with TH uptake by the hypothalamus or pituitary may be one of the mechanisms.

Alternatively, a sorafenib-induced decrease in deiodinase type 2 activity in the hypothalamus or pituitary (described later) would have similar consequences on the HPT axis setpoint. Alternative explanations could be interference with binding of T3 to its nuclear receptor, a diminished pituitary blood flow due to the antiangiogenic effects of sorafenib (33), or a reduced clearance of TSH (34). Future studies should investigate which mechanisms contribute to this altered sensitivity of the HPT axis.

Peripheral TH metabolism

There was a marked decrease in the serum T3/rT3 and T3/T4 ratios, suggesting additional changes in the peripheral metabolism of TH because

an isolated change in the HPT axis setpoint would not necessarily affect these ratios.

In previous experiments, we showed sunitinib-induced D3 activity in normal rat liver (33). The decreased T3/rT3 ratio observed in the current study fits with an induction of TH inactivation by D3, as has been described by Abdulrahman *et al.* (16). In the case of constant T4 production, increased D3 activity would not only lead to increased rT3 production but also to a decrease in T4 levels. However, in contrast to the study of Abdulrahman *et al* (16), which analyzed thyroidectomized patients on TSH-suppressive doses of levothyroxine, our study was performed in patients with intact thyroid glands. We did not only see effects on peripheral metabolism of TH but also a central effect on the pituitary, with an increase in TSH that would increase T4 production. This likely explains why T4 increases despite the decreased T3/rT3 ratio, which we assume to be caused by an increase in D3 activity. Similarly, the decreased T3/T4 ratio fits with a lower T4 to T3 conversion. The higher T4 production may explain why T3 levels remain stable despite this lower T4 to T3 conversion.

The increase in FT4 and the subsequent decrease in T3/T4 might suggest that other peripheral TH-metabolizing enzymes are also affected, such as a decrease in deiodinase type 1 or deiodinase type 2. In addition, uptake of TH into cells is rate limiting for subsequent metabolism. Because MCT8 is not only expressed in the brain but also in multiple other tissues (35) and MCT10 is highly expressed in skeletal muscle, kidney, pancreas, and intestine (32) sorafenib-induced inhibition of T3 uptake by MCT8 and MCT10 may also have contributed to the alterations in peripheral metabolism of TH. Further experiments are needed to investigate the contribution of these different mechanisms in more detail.

Nonthyroidal illness (NTI) is not likely to be a major contributor because (1) changes in T3/rT3 and T3/T4 were most evident in the first weeks after start of treatment and not at the end of study (when the cancer was progressive) and (2) TSH and FT4 may remain normal in mild NTI, but the persistent and progressive increase in TSH and FT4 does not fit the pattern of NTI (36).

The changes in TH metabolism are independent of changes in binding proteins. A decrease in TH binding proteins would have led to a similar decrease in all iodothyronines, whereas we did see marked changes in the total T3/rT3 and rT3/T4 ratios, in which the binding proteins are both in the numerator and the denominator (37). Furthermore, measurements of FT4 levels and total T4 levels changed in the same direction.

Clinical outcome

In this study, we found that an increase in TSH is an independent negative prognostic marker for PFS. There was no association between FT4 and survival. *In vivo*, local hypothyroidism is known to be associated with HCC progression (38). In patients with other solid tumors, especially basal cell carcinoma, induction of D3 is associated with a hyperproliferative state and carcinogenesis (39). However, our results seem to be in contrast with two other studies in patients treated with sorafenib or sunitinib, where an increased TSH was negatively associated with tumor progression (10, 13). It is not yet clear how to explain this inconsistency between these studies. However, there are a few differences between the studies: (1) in the previous studies, only patients with TSH levels above the normal range were investigated, whereas we assessed the absolute change of TSH on PFS and survival in all patients (including patients within the reference range); (2) in the previous studies, patients received levothyroxine, which may have affected tumor progression and makes it difficult to compare the results; and (3) in the other studies, patients with thyroiditis, who might have different prognostic profiles, were not excluded. Future studies are therefore needed to unravel if and how the effects of sorafenib on thyroid function can be regarded as a prognostic factor.

CONCLUSIONS

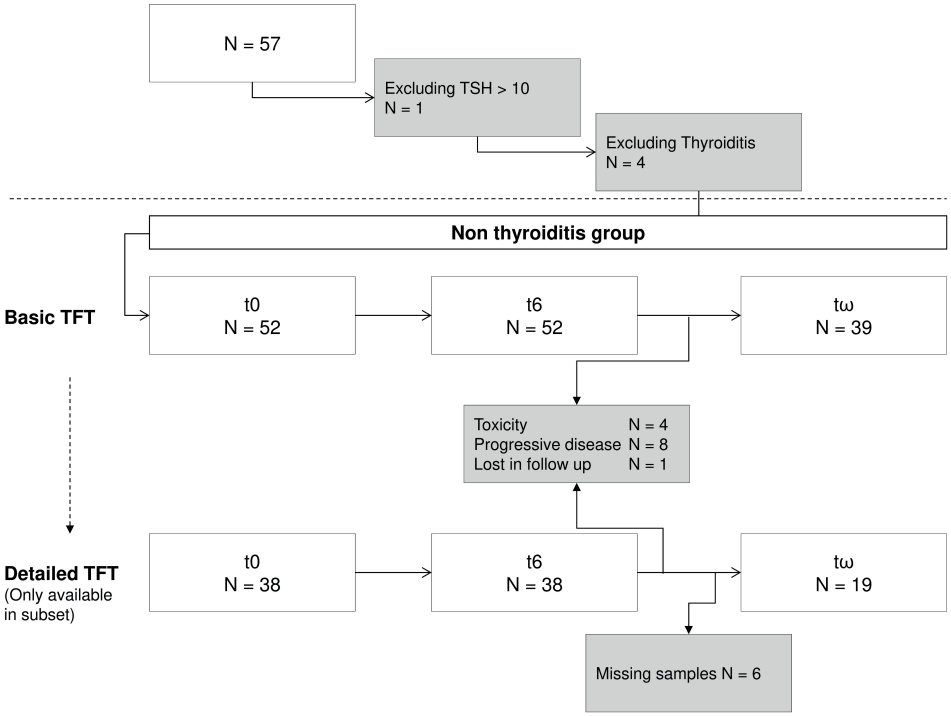
Our clinical data demonstrate that sorafenib-induced changes in TFTs are mediated at several levels, with *in vitro* experiments showing sorafenib-induced inhibition of T3 transport into the cell by MCT8 and MCT10.

Acknowledgments

We thank Esther Oomen-de Hoop for statistical advice.

Disclosure Summary

The authors have nothing to disclose.



Supplemental Figure 1.

Supplemental Table 1.

	Delta TSH	*p*	Delta FT4	P
Prognosis HR (CI95%)				
Death	1.16[0.98-1.37]	0.09	1.02[0.94-1.10]	0.79
Progression	1.26[1.04-1.53]	0.001**	0.96[0.87-1.04]	0.31
Adverse events OR (CI95%)				
HFSR	1.14[0.76-1.70]	0.53	0.92[0.80-1.06]	0.26
Hypertension	1.00[0.56-1.76]	0.99	1.00[0.81-1.24]	0.97
Severe liver toxicity	0.74[0.49-1.12]	0.15	1.05[0.89-1.23]	0.59
Gastrointestinal	1.40[0.75-2.62]	0.29	0.92[0.78-1.08]	0.28
Thrombocytopenia	0.82[0.53-1.27]	0.37	0.97[0.85-1.12]	0.69

Adjusted hazard ratios (HR) were calculated in a cox proportional-hazard regression model adjusted for age, gender, world health organization performance status and average dose of sorafenib.

CI 95%, 95% confidence interval; Delta FT4, FT4 t6-FT4 t0; Delta TSH, TSH t6-TSH t0; HFSR, hand foot skin reaction; OR, odds ratio; t0, before treatment; t6, after 6 weeks of treatment; *, p<0.05; ** , p<0.01

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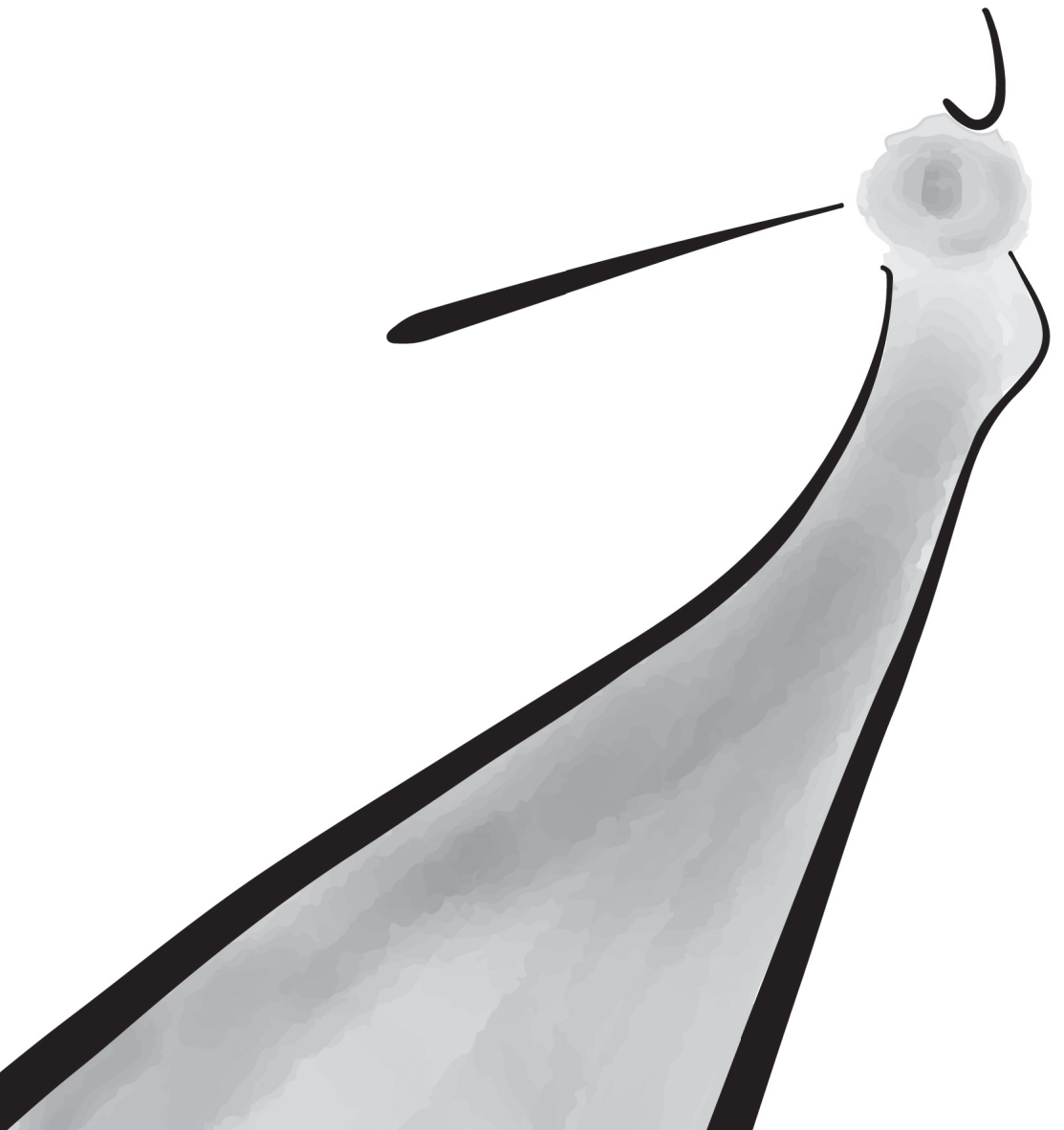

CHAPTER 4

Effects of Thyrotropin on Peripheral Thyroid Hormone Metabolism and Serum Lipids

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ABSTRACT

Background

Subclinical hypothyroidism is associated with dyslipidemia and atherosclerosis. Whether these effects are in part mediated via direct effects of thyrotropin (TSH) on peripheral thyroid hormone (TH) metabolism and/or concentrations of serum lipids is not clear.

Objective

This study examined whether TSH has direct effects on peripheral TH metabolism and serum lipids.

Methods

Eighty-two patients with differentiated thyroid cancer were retrospectively analyzed. All patients had undergone total thyroidectomy and ^{131}I remnant ablation. During follow-up, two successive injections of recombinant human TSH (rhTSH) were administered to patients on a stable dose of levothyroxine. In all patients, TSH, thyroxine (T4), free T4 (fT4), triiodothyronine (T3), reverse T3 (rT3), total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, apolipoprotein B, lipoprotein(a), and triglyceride levels were measured immediately before the first and approximately 72 hours after the second injection of rhTSH.

Results

After rhTSH stimulation, T3 values decreased (from 1.91 to 1.81 nmol/L; $p < 0.001$). T4, fT4, and rT3 did not change. After rhTSH, median apolipoprotein B increased from 0.90 to 0.92 g/L ($p = 0.03$), lipoprotein(a) from 0.21 to 0.24 g/L ($p < 0.001$), and triglycerides from 1.98 to 2.50 mmol/L ($p < 0.001$). Serum high-density lipoprotein cholesterol decreased from 0.98 to 0.81 mmol/L ($p < 0.001$). Multiple regression analysis showed that the changes in lipids were most closely associated with the decrease in T3 levels.

Conclusions

TSH has direct effects on peripheral TH metabolism by decreasing T3 levels in levothyroxine-treated thyroidectomized patients. This decrease in T3 levels is accompanied by unfavorable changes in serum lipids.

Abbreviations

apoB, apolipoprotein B; DTC, differentiated thyroid carcinoma; D1-3, deiodinase type 1-3; FT4, free thyroxine; FT3, free triiodothyronine; HDL-C, high-density lipoprotein cholesterol; IQR, interquartile range; LDL-C, low-density lipoprotein cholesterol; Lp(a), lipoprotein(a); LT4, levothyroxine; TgAb, thyroglobulin antibodies; TH, thyroid hormone; TFT, thyroid function test; TSH, thyroid stimulating hormone; rhTSH, recombinant human TSH; T3, triiodothyronine; T4, thyroxine; rT3, reverse triiodothyronine; VLDL-C, very-low-density lipoprotein

INTRODUCTION

Subclinical hypothyroidism is defined by an elevated thyrotropin (thyroid stimulating hormone; TSH) with thyroxine (T4) and triiodothyronine (T3) levels within the normal range. This condition is associated with increased cardiovascular risk and elevated levels of total and low-density lipoprotein cholesterol (LDL-C) (1-7). It is likely that this association is caused by the slightly lower thyroid hormone (TH) levels in patients with subclinical hypothyroidism, rather than by the elevated TSH itself (8, 9). However, extrathyroidal TSH receptors have been shown in extraocular muscle, bone, fat tissue and liver, indicating that TSH may also have extrathyroidal effects (10-12). Studies in liver cells and rats suggest that TSH directly upregulates cholesterol synthesis via increased hepatic expression of 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (11, 12). This led to the hypothesis that part of the effects of subclinical hypothyroidism on lipid levels may also be mediated via direct effects of TSH.

Approximately 20% of circulating T3 in healthy subjects is secreted by their thyroid gland. The major fraction is produced by deiodination of T4 in peripheral tissues (13). Intracellular T4 is converted to bioactive T3 by type 1 and type 2 deiodinase (D1, D2). Inactivation of TH is mediated mainly by type 3 deiodinase (D3), which converts T4 to reverse T3 (rT3) and T3 to 3,3'-diiodothyronine (14). Many factors have been identified that influence peripheral TH metabolism, such as starvation, medication, illness, and long-term TSH suppressive (LT4) therapy (15, 16). Although TSH has been shown to increase hepatic conversion of T4 to T3 in perfused rat liver (17), direct effects of TSH on peripheral TH metabolism have not been reported in humans.

Patients treated for differentiated thyroid cancer (DTC) by thyroidectomy and ^{131}I ablation completely lack functional thyroid tissue. Consequently, all serum T3 in these patients results from conversion of T4 in peripheral tissues. In the follow-up of DTC, most patients may undergo a recombinant human TSH (rhTSH) stimulation test as part of a dynamic risk stratification. This stimulation test provides an ideal model to study the effects of TSH because of the constant T4 supply. The present study took advantage of this model to determine whether TSH has direct effects on peripheral TH metabolism and serum lipid levels.

METHODS

Patients and treatment

Initial therapy consisted of total thyroidectomy and radioiodine (^{131}I) remnant ablation. Patients were on TSH suppressive LT4 therapy aiming at a TSH level ≤0.1 mU/L. Success of ablation was evaluated after about 6 months by means of rhTSH stimulated thyroglobulin measurements. On day 1, baseline non-fasting blood was drawn for measurement of thyroid function tests (TFT) and lipids. Subsequently, 0.9 mg of rhTSH was administered intramuscularly on days 1 and 2. On day 5, a second nonfasting blood sample was drawn.

Data of the rhTSH tests with a negative thyroglobulin response and negative thyroglobulin antibodies (TgAb) were retrospectively analyzed. The present study employed serum samples collected in an ethically approved study at the Erasmus MC (MEC 2012-561). Furthermore, leftover remnant sera from regular clinical procedures from the University Hospital Wuerzburg from the 2009–2013 time frame were analyzed. The study was performed in accordance with the German regulations on the use of serum sample remnants after diagnostic use. Samples were anonymized before analysis.

TFT

TFT and lipid levels before and after rhTSH administration were analyzed in the same run. Serum TSH (reference range 0.4–4.3 mIU/L), free T4 (fT4; reference range 11–25 pmol/L), thyroglobulin, and TgAb were measured as part of routine clinical practice. In Erasmus MC, TSH, thyroglobulin, and TgAb were measured using the Immulite 2000XPi platform (Siemens, Los Angeles, CA) with functional sensitivities of 0.01 mIU/L for TSH, 0.2 lg/L for thyroglobulin, and 2.2 IU/mL for TgAb. fT4 was measured using the Vitros ECiQ(Ortho-Clinical Diagnostics, Rochester, NY), with a functional sensitivity of 0.88 pmol/L. At the University Hospital Wuerzburg, TSH, fT4, as well as free T3 (fT3; reference range 2.7–7.6 pmol/L) were also measured using the Immulite 2000XPi platform (Siemens). The intra-assay coefficient of variation for fT3 was 3.2–7.0%. Serum thyroglobulin levels were determined using the immunoradiometric assay from BRAHMS (functional sensitivity of 0.4 lg/L) (9). TgAb levels were assessed using the direct chemoluminometric VARELISA method (Thermo Fisher Scientific, BRAHMS, Uppsala, Sweden). In all samples, total T4 (reference range 58–128 nmol/L) and T3 levels (reference range 1.43–2.51 nmol/L) were measured using the Vitros ECiQ (Ortho-Clinical Diagnostics). Reverse T3 (reference range 0.21–0.54 nmol/L) was measured with an in-house radioimmunoassay (18) with an intra-assay coefficient of

variation of 4.2–8.7%. T3/T4 x100 (reference range 1.42–3.05), T3/rT3 (reference range 2.65–7.65), and rT3/T4 x100 (reference range 0.15–0.44) ratios were calculated. These ratios are relatively insensitive to variations in protein binding and are considered to reflect peripheral deiodinase activities (19).

Lipids

Direct measurement of total cholesterol (reference range 2.9–6.5 mmol/L), LDL-C (reference range 2.59–4.12 mmol/L), high-density lipoprotein cholesterol (HDL-C; reference range 0.9–1.1 mmol/L), and triglycerides (reference range <2.0 mmol/L) were measured using standard laboratory techniques. Apolipoprotein B (apoB; reference range 0.60–1.33 g/L) was measured by immunoturbidimetry on a c311automatic analyzer (Roche Diagnostics, Basel, Switzerland). Plasma lipoprotein(a) (Lp(a); reference range <0.30 g/L) concentrations were measured using a particle-enhanced immunoturbidimetric assay, which is largely independent of the apo(a) kringel IV type 2 repeat number (DiaSys Diagnostic System, GmbH, Holzheim, Germany) (20). LDL-C was also calculated via the Friedewald formula [total cholesterol-HDL-C-(0.45 x triglycerides)] (21) and non-HDLC via the formula (total cholesterol – HDL-C) (21).

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows v23 (IBM Corp., Armonk, NY). Changes in TFT and lipids were analyzed using the paired-sample t-test, and if not normally distributed via the paired Wilcoxon signed-rank test. Power calculations showed that with the largest sample that was available, using an alpha of 0.05 and a power of 80%, a statistically significant effect size of 0.3 would be able to be detected (22). Normal distribution was ascertained using the Kolmogorov–Smirnov test, and residuals that were not normally distributed were log transformed. Multiple linear regression models were used to study if the association of TSH with serum lipids was (partly) mediated via changes in T3. Multiple comparisons were accounted for using the Benjamini and Hochberg false discovery procedure (23). Corrected p-values of <0.05 were considered significant.

RESULTS

A total of 82 patients were studied: 56 patients from the University Hospital Wuerzburg and 26 patients from the Erasmus MC, Rotterdam. Thirty-two

patients were male. The median age was 44 years (interquartile range [IQR] = 35–59 years). None of the patients used medication that may influence TH metabolism (e.g., corticosteroids or amiodarone).

TH

As expected, serum TSH concentrations increased strongly after rhTSH injection (Table 1). Despite a constant dose of LT4 and stable concentrations of fT4 and rT3, T3 concentrations decreased after rhTSH injections (Table 1). The decrease in T3/T4 ratio and T3/rT3 ratio was mainly due to the decrease in T3 values (Table 1). Additionally, serum FT3, which was measured in the clinical routine in the 51 patients included in Wuerzburg, also decreased (Table 1).

Table 1. Change in thyroid function tests after recombinant TSH stimulation

Variable	*reference value*	*before rhTSH*	*after rhTSH*	*p*
Parameter				
TSH (mU/L)¥	0.4-4.3	0.02 [0.01-1.00]	9.68 [7.30-14.6]	<0.001***
FT4 (pmol/L)£	11-25	23.6 (4.7)	23.6 (4.9)	0.41
T4 (nmol/L) £	58-128	156 (41)	159 (43)	0.41
T3 (nmol/L) £	1.43-2.51	1.91 (0.30)	1.81 (0.30)	<0.001***
FT3 (pmol/L) £	2.7-7.6	5.71 (1.34)	5.01 (1.05)	<0.001***
rT3 (nmol/L) £	0.21-0.54	0.53 (0.10)	0.53 (0.20)	0.90
Ratio				
T3/T4x100¥	1.42-3.05	1.22 [1.01-1.44]	1.13 [0.91-1.47]	<0.001***
T3/rT3¥	2.65-7.65	3.61 [2.96-4.63]	3.53 [2.75-4.34]	0.02**
rT3/T4x100¥	0.15-0.44	0.33 [0.30-0.40]	0.33 [0.29-0.39]	0.90

Changes in TFT were analyzed using a paired sample *t* test and if not normally distributed via a paired Wilcoxon signed-rank test. False discovery rate correction for multiple comparisons was applied as proposed by Benjamini and Hochberg.

£,Mean (SD); ¥Median [IQR]; ** , $p \leq 0.01$; ***, $p \leq 0.001$

FT4, free thyroxine; IQR, interquartile range; SD, standard deviation; TFT, thyroid function test; TSH, thyroid stimulating hormone; rhTSH, recombinant TSH; T3, triiodothyronine; T4, thyroxine; rT3, reverse T3

Lipids

Median apoB, Lp(a), non-HDL-C and triglycerides increased after rhTSH. Serum HDL-C decreased, whereas total cholesterol and LDL-C did not change (Table 2).

Table 2. Changes in non-fasting serum lipid concentrations after recombinant human TSH injection

Variable	*reference value*	*before rhTSH*	*after rhTSH*	*p*
apoB (g/L) [£]	0.60-1.33	0.90 (0.03)	0.92 (0.03)	<0.05*
Total cholesterol (mmol/L) [£]	2.9-6.5	5.09 (0.14)	5.17 (0.13)	0.17
HDL-C (mmol/L) [£]	0.9-1.1	0.98 (0.05)	0.81 (0.05)	<0.001***
LDL-C (mmol/L) [£]	2.59-4.12	2.90 (0.10)	2.84 (0.10)	0.40
Lp(a) (g/L) [£]	<0.30	0.21 (0.03)	0.24 (0.03)	<0.001***
Triglycerides (mmol/L) [£]	<2.0	1.98 (0.14)	2.50 (0.16)	<0.001***
LDL-C-Friedewald (mmol/L) [£]	2.59-4.12	3.24 (0.13)	3.29 (0.13)	0.40
Non-HDL-C (mmol/L) [£]	2.0-5.4	4.10 (0.15)	4.36 (0.15)	<0.001***

Analysis by general linear model for repeated measurements was used and false discovery rate correction for multiple comparisons proposed by Benjamini and Hochberg was applied.

Friedewald formula [total cholesterol-HDL-C-(0.45 x triglycerides); non-HDL-C formula (total cholesterol-HDL-C); [£],Mean (SD); * , $p \leq 0.05$; ***, $p \leq 0.001$

apoB, Apolipoprotein B; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; Lp(a), Lipoprotein(a); SD, standard deviation; rhTSH, recombinant human TSH

Next, to determine if these changes in lipid levels could be attributed to direct effects of rhTSH or to its effects on serum T3 levels, serum T3 levels were added as a covariate to the multiple regression model. This showed that the changes in lipid levels were most closely associated with the changes in T3 and showed no evidence for an additional effect of rhTSH itself (Table 3).

Table 3. T3 adjusted change in non-fasting serum lipid concentrations after recombinant human TSH stimulation

Variable	*reference value*	*before rhTSH*	*after rhTSH*	*adjusted p*
apoB (g/L) [£]	0.60-1.33	0.90 (0.03)	0.92 (0.03)	0.74
Total cholesterol (mmol/L) [£]	2.9-6.5	5.09 (0.14)	5.17 (0.13)	0.74
HDL-C (mmol/L) [£]	0.9-1.1	0.98 (0.05)	0.81 (0.05)	0.92
LDL-C (mmol/L) [£]	2.59-4.12	2.90 (0.10)	2.84 (0.10)	0.92
Lp(a) (g/L) [£]	<0.30	0.21 (0.03)	0.24 (0.03)	0.74
Triglycerides (mmol/L) [£]	<2.0	1.98 (0.14)	2.50 (0.16)	0.92
LDL-C-Friedewald (mmol/L) [£]	2.59-4.12	3.24 (0.13)	3.29 (0.13)	0.74
Non-HDL-C[£]	2.0-5.4	4.10 (0.15)	4.36 (0.15)	0.74

Multiple regression analysis was performed in general linear model for repeated measurements, where changes in serum lipids were corrected for change in T3. False discovery rate correction for multiple comparisons was applied as proposed by Benjamini and Hochberg.

Friedewald formula [total cholesterol-HDL-C-(0.45 x triglycerides); non-HDL-C formula (total cholesterol-HDL-C); [£],Mean (SD)

apoB, Apolipoprotein B; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; Lp(a), Lipoprotein(a); SD, standard deviation; rhTSH, recombinant human TSH; T3, triiodothyronine

DISCUSSION

To the best of the authors' knowledge, this is the first study that provides evidence of a direct effect of TSH on peripheral TH metabolism in humans. The study was performed in a controlled setting during which patients were on a stable daily LT4 dosage. Administration of exogenous rhTSH led to a decrease in T3 and fT3 concentrations, and as a consequence also to decreased T3/T4 and T3/rT3 ratios. The results indicate that any TSH-related changes in lipid levels *in vivo* are mediated by a drop in (f)T3 concentrations when T3 is added as a covariate to the multiple regression model.

Peripheral TH metabolism

Unravelling the exact molecular mechanism causing the decreased T3 concentrations requires further *in vitro* and *in vivo* studies. Potentially, the decrease in T3 concentrations can be explained by a decreased conversion of T4 to T3 by D1 or D2, or by an increased degradation of T3 to rT3 by D3. Since D2 may be more important than D1 for peripheral T3 production in humans, whereas D1 may be more important than D2 for rT3 degradation (24), it is speculated that a decrease in D2 activity is the most likely explanation for this reduction in T3 levels in the presence of unaltered rT3 levels. In the case of decreased D1 activity, an increase in rT3 concentrations would be expected as well (19, 25). An increased degradation by D3 would not only lead to lower concentrations of T3, but also to lower concentrations of T4 given the constant LT4 dose, and an increase in rT3 concentrations (via the degradation of T4).

Other explanations for the decrease in T3 concentrations upon rhTSH stimulation include an increased T3 degradation via nondeiodinative conjugation by forming T3 sulfate (26) and/or T3-glucuronide formation (27). These reactions would increase T3 solubility and biliary and urinary excretion. Also, altered T4 transport into T3 producing cells could explain the decrease in T3 concentrations, since this transport is the ratelimiting step for subsequent metabolism.

Most circulating T3 is bound to proteins such as thyroxinebinding globulin, albumin, and transthyretin. Given that not only total T3, but also FT3 concentrations decreased after rhTSH administration, it seems unlikely that the drop in serum T3 concentrations after rhTSH stimulation is caused by a change in T3 binding protein concentrations or affinity.

A temporary change in diet after rhTSH injections might theoretically result in lower T3 levels (14), since fasting is a known cause of low T3 syndrome.

However, caloric restriction would decrease especially triglycerides (28). The increase in serum triglycerides that was identified and the lack of change in rT3 make this explanation highly unlikely. Furthermore, as most patients in this study underwent the procedure in an in-patient setting due to German nuclear medicine regulations, it was ascertained that patients did not fast between the two blood samples. A previous study in 15 DTC patients, did not show an effect of rhTSH administration on serum FT4 and FT3 concentrations, perhaps due to a lack of statistical power (29).

Lipids

In the current study, short-term exposure to high TSH concentrations was associated with a decrease in (F)T3 concentrations and subsequently, a more unfavorable serum lipid profile.

Hypothyroidism-induced changes in serum lipids are well established and characterized by elevated total cholesterol (6, 30), LDL-C (6, 31), triglycerides (32), Lp(a) (33), apoB (33) and decreased HDL-C (34). These changes are mediated via changes in cholesterol biosynthesis (3-hydroxy-3-methylglutaryl-coenzym A), regulation of LDL-receptors (via sterol regulatory element-binding protein 2) (35), HDLmediated reverse cholesterol transport to the liver (36) and biliary cholesterol excretion (37, 38). The beneficial effect of TH on the lipid profile has led to the development of "liverspecific" thyromimetics such as Eprotirome. Eprotirome affects the T3 receptor-β isoform and results in a decrease in LDL-C, apoB, triglycerides, and Lp(a) (39). The current study, identified a decrease in T3 and a very similar diametrically opposite effect in apoB, triglycerides, and Lp(a), which seems to support a diminished T3 stimulation.

LDL-C concentrations did not significantly change in this study, but non-HDL-C as well as apoB increased indicating increased levels of triglyceride-rich lipoproteins such as very -low-density lipoprotein (VLDL) or intermediatedensity lipoprotein. In line with this observation, others have shown a significant elevation of VLDL-C in patients with hypothyroidism (40).

Potentially, the nonfasting status had an impact on these results. However, the identified changes in lipids are much larger and not in line with extensive observational data as summarized in the European Society of Cardiology guidelines (41). The most important differences are that the nonfasting status affects triglycerides, with an increase of 0.3 mmol/L versus an increase of 0.5 mmol/L in the current study, and the calculated non-HDL-C decreases by 0.2 mmol/L compared to an increase of 0.3 mmol/L in the current study.

Moreover, concentrations of HDL-C, apoB, and Lp(a) are not affected by fasting/nonfasting status in contrast to the current data.

In the current study, all patients lacked functional thyroid tissue due to surgery and radioiodine ablation therapy. Therefore, the isolated effects of rhTSH were able to be studied in a very homogenous group with a constant LT4 supply. However, this study also has several limitations. First of all, in contrast to the short serum half-life of triglycerides (42) and VLDL-C (43) of only a few hours, the half-life of the other lipoproteins is as long as several days (44, 45). As a consequence, the steady state of serum concentration of most lipoproteins was not yet reached in this study. A maximal effect would probably be found after two weeks (46, 47), but the clearance rate and the effect of two injections of rhTSH versus continuous TSH stimulation are difficult to predict. The timeframe of five days was, however, sufficient to identify significant changes in the current study. A significant change in LDL clearance can occur within one day through a change in the activities of hepatic microsomal cholesterol 7alpha-hydroxylase and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (48). Changes in Lp(a) concentrations over several days have been shown to be mainly determined by alterations in the rate of production in a kinetic study with radiolabeled Lp(a) (44).

Second, statistical modeling can only give insight into possible associations. In other experiments, a direct effect of TSH on serum lipids has been suggested, but a possible confounding effect of a decrease in T3 concentrations has not been investigated (12, 49, 50). Further research with direct testing is necessary to unravel fully the pathophysiologic mechanisms of TSH and TH action on lipid metabolism.

Third, the current study was performed in a specific patient population without a control group, and the data were, in part, collected retrospectively. Moreover, the possibility cannot formally be excluded that the rhTSH injections did not result, for example, in cytokine release and secondary changes in TH metabolism and action (51). Therefore, further studies in larger placebo-controlled cohorts including individuals other than thyroid cancer patients are required.

CONCLUSION

RhTSH administration results in a decrease of serum T3 concentrations in LT4-treated thyroidectomized patients, which in turn affect serum lipid concentrations toward a more unfavorable profile. These data suggest a direct effect of TSH on peripheral T3 production. Replication is needed, and

future studies should further clarify by which molecular mechanisms TSH affects peripheral TH metabolism.

AUTHOR DISCLOSURE STATEMENT

F.A.V. is a consultant to Bayer Healthcare and Sanofi Genzyme and has received speaker honoraria from Genzyme and Diasorin. R.P.P. has received lecture and consultancy fees from Sanofi Genzyme, and lecture fees from Goodlife Fertility BV and IBSA (Institute Biochemique SA). No competing financial interests exist for the remaining authors.

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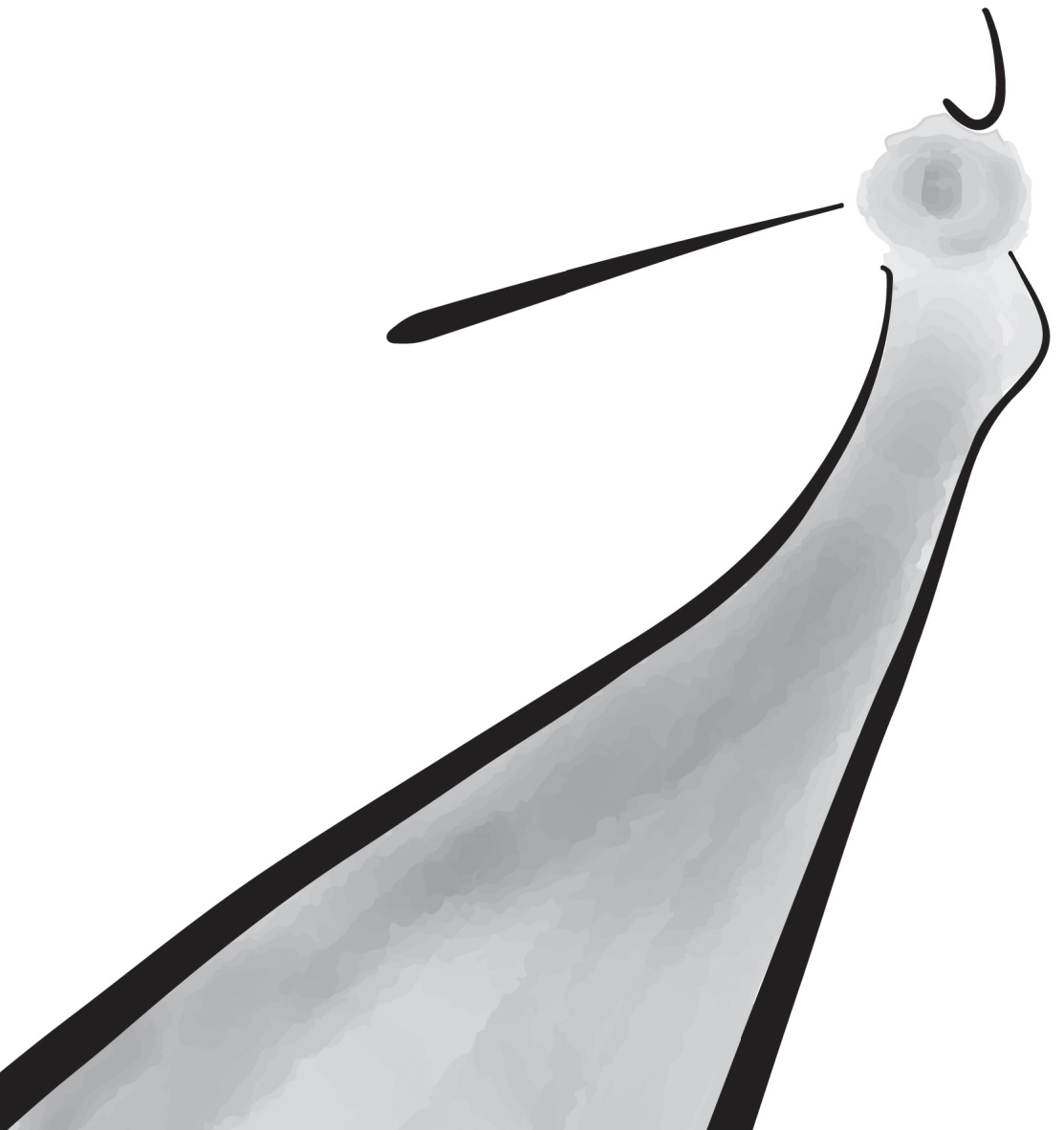

CHAPTER 5

Effects of Thyroid Hormone on Urinary Concentrating Ability

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ABSTRACT

Background

Hypothyroidism has been associated with impaired urinary concentrating ability. However, previous reports on thyroid hormone and urinary concentrating ability in humans only studied a limited number of patients with autoimmune thyroid disease or used healthy controls instead of paired analysis within the same patients.

Objective

To study the urinary concentrating ability in athyreotic patients with differentiated thyroid cancer on and off levothyroxine treatment as they are exposed to different thyroid states as part of their treatment in the absence of an autoimmune disease.

Design and methods

We studied 9 patients (mean age of 42.7 years) during severe hypothyroid state (withdrawal of levothyroxine before radioactive iodine therapy) and TSH suppressed state (on levothyroxine therapy). At these two points, serum and urine samples were collected after 14 hours of overnight thirsting.

Results

Serum and urine osmolality were not significantly different between on and off levothyroxine treatment. Serum creatinine levels were significantly higher in patients off vs. on levothyroxine treatment (87.0 µmol/L vs. 71.0 µmol/L respectively; $p = 0.044$) and, correspondingly, the estimated glomerular filtration rate (eGFR) was significantly lower (89.6 mL/min vs. 93.1 mL/min respectively; $p = 0.038$).

Conclusion

Short-term, severe hypothyroidism has no effect on urinary concentrating ability. Our study confirms the well-known effects of thyroid hormone on serum creatinine concentrations.

INTRODUCTION

Thyroid hormone (TH) is indispensable for the metabolism of all tissues. The importance of TH in normal physiology is well illustrated by primary thyroid diseases in which abnormal TH concentrations affect the function of several organs resulting in a variety of clinical symptoms (1).

The ability to conserve water during periods of water deprivation is an important function of the kidney. Fluid deprivation increases serum osmolality and thereby causes a release of the antidiuretic hormone (arginine vasopressin (AVP)). In turn, in the principal cells of the collecting duct, vasopressin inserts pre-formed vesicles with the water channel aquaporin-2 into the apical plasma membrane to allow water reabsorption (2). The counter current mechanism creates the osmotic driving force for water reabsorption in the collecting duct.

Hypothyroidism has been associated with impaired urinary concentrating capacity in animals and humans (3-6). Short-term hypothyroidism in rats results in a diminished medullary osmotic driving force for passive water movement across the collecting duct. This was associated with a significant decrease in the medullary sodium potassium chloride cotransporter type 2 (NKCC2) (4). The impaired maximal urinary concentrating capacity in these rats with moderate hypothyroidism was readily reversed with TH replacement. On the other hand, long term hypothyroidism in rats resulted in an impaired urine dilution capacity after water loading as a result of the non-osmotic release of vasopressin (3). This defect was reversed by administering a vasopressin receptor antagonist.

Only a few studies on the urinary concentrating defect in hypothyroidism have been performed in patients with autoimmune thyroid disease (5, 6). A small study (n=4) of patients with hypothyroidism revealed a urinary concentrating defect after 16 hours of water deprivation (5). After adequate treatment with levothyroxine (LT4), this defect was corrected. Another study found similar defects in myxoedema patients (n=10) compared to healthy control subjects (n=15) after 16 hours of water restriction (6). Treatment of a small group of these patients (n=3) showed no improvement in urinary concentrating capacity. These studies on TH and urinary concentrating ability in humans only studied a very limited number of patients (5) or used healthy controls instead of paired analysis within the same patients (6). Furthermore, detailed thyroid function tests were not performed.

The aim of this study was to extend the existing studies by investigating the urinary concentration ability for the first time in athyreotic patients, before

and after LT4 treatment. We therefore studied patients with differentiated thyroid cancer (DTC), as they are exposed to different thyroid states as part of their treatment in the absence of autoimmune disease.

SUBJECTS AND METHODS

DTC patients, 18-65 years old, were recruited from the outpatient clinic of the Erasmus Medical Center Rotterdam, between November 2014 and October 2015. Initial therapy consisted of total thyroidectomy. Patients were eligible for inclusion if they were scheduled for treatment with radioactive iodine (RAI); did not use drugs interfering with TH metabolism or drugs influencing urinary concentration capacity (e.g, diuretics, lithium, non-steroidal anti-inflammatory drugs); did not have a urinary tract infection; had no history of diabetes insipidus, diabetes mellitus or adrenal insufficiency; and had an estimated glomerular filtration rate (eGFR) > 60 mL/min per 1.73 m2. Patients were instructed to restrain from water and food for 14 hours before their outpatient visit on two different occasions. The first measurement was scheduled after four weeks of TH withdrawal (before RAI therapy, to stimulate radioactive iodine uptake by malignant tissues) and the second a few months after restoring euthyroidism / TSH suppression. Our primary endpoint was the difference in urine osmolality between LT4 withdrawal and treatment. Peripheral blood samples and spot urine samples were obtained from all participants. The Medical Ethics Committee of the Erasmus Medical Center approved the study protocol (MEC-2014-134) and written informed consent was obtained from all study participants.

Laboratory measurements

Serum Free T4 (FT4) (reference range 11.0-25.0 pmol/L), total T4 (reference range 58.0-128.0 nmol/L) and total T3 (reference range 1.4-2.5 nmol/L) concentrations were measured by chemo luminescence assays (Vitros ECI Immunodiagnostic System; Ortho-Clinical Diagnostics, Rochester, MI). Serum TSH (reference range 0.4-4.3 mU/L) was measured by immunometric assay (Immulite 2000 XPi, Siemens, The Hague, the Netherlands). Serum and urine osmolality were measured by OM 6050 Osmo Station from A. Menarini Diagnostic and serum sodium, potassium, urea, creatinine, chloride and glucose concentrations were measured by Roche/Hitachi cobas c systems. The eGFR was computed automatically with the Chronic Kidney Disease Epidemiology Collaboration formula (CKD-EPI). Sodium, urea and creatinine concentrations were also measured in urine samples by Roche/Hitachi cobas c systems.

Statistical analysis

Based on the previous studies (5, 6), we postulated that a study in 10 patients on and off LT4 treatment would be of sufficient sample size to find a significant difference in urine osmolality. Data were expressed as median with 25th and 75th percentiles. For paired analysis between patients on and off LT4 treatment the Wilcoxon signed rank test was used. We used SPSS 20.0 for Windows (SPSS, Inc., Chicago, IL, USA). The spearman rank correlation coefficient was calculated to evaluate the correlation between urine osmolality and age and thyroid function tests off and on LT4 treatment. A p-value < 0.05 was considered as statistically significant.

RESULTS

We included 10 patients in the study. However, one of them started on treatment with a diuretic after the first visit and was therefore excluded. Nine patients with a mean age of 42.7 years [range 24-57 years] were analysed (Table 1). As expected, thyroid function tests were significantly different on and off LT4 treatment (Table 2), reaching very low levels of FT4 (median 1.7 pmol/L) off LT4. Serum creatinine levels were significantly higher (87.0 vs. 71.0 µmol/L, p = 0.044) and the eGFR was significantly lower in hypothyroid state than in LT4 treated state (89.6 mL/min vs. 93.1 mL/min respectively; p = 0.038). Serum glucose levels were significantly lower during hypothyroidism (4.8 vs. 5.3 mmol/L, p = 0.011). Serum sodium and chloride levels were significantly higher during LT4 treatment than during hypothyroidism (143 vs. 141 mmol/L; p = 0.011 and 104.0 vs. 99.0 mmol/L; p = 0.007, respectively), while serum osmolality remained similar (287.0 on LT4 vs. 282.0 mOsm/kg off LT4; p = 0.09).

Table 1. Characteristics of study participants

Sex N (%)	
Male	4 (44.4)
Female	5 (55.6)
Age (years), mean (± SD)	42.7 (± 11.0)
Time between tests(days), median [25th-75th percentile]	124 [91-161]
Dose LT4 (µg), mean [range]	204.2 [150-325]
Dose LT4 (µg/kg), mean [range]	2.27 [1.6-3.1]
Diagnosis N (%)	
Papillary thyroid cancer	7 (82.5)
Follicular thyroid cancer	2 (12.6)

Table 2. Changes in thyroid function tests and serum electrolytes, creatinine and osmolality off and on LT4 treatment

	Off LT4		LT4 treated		p-value
TSH (mU/L)	68.4	[42.0-102.5]	0.049	[0.005-0.57]	0.008
FT4 (pmol/L)	1.7	[1.2-3.4]	24.1	[21.5-25.3]	0.008
Total T4 (nmol/L)	17.0	[14.0-28.0]	146.0	[132.0-170.5]	0.008
Total T3 (nmol/L)	0.7	[0.6-1.0]	2.1	[2.0-2.3]	0.008
Creatinine (µmol/L)	87.0	[76.5-92.0]	71.0	[67.5-89.5]	0.044
eGFR (mL/min)	89.6	[66.4-93.1]	93.1	[85.8-103.8]	0.038
Urea (mmol/L)	4.6	[3.7-5.1]	4.8	[4.1-5.2]	0.21
Sodium (mmol/L)	141.0	[138.5-141.5]	143.0	[142.0-144.5]	0.011
Glucose (mmol/L)	4.8	[4.5-5.1]	5.3	[4.9-5.6]	0.011
Potassium (mmol/L)	4.3	[4.2-4.5]	4.4	[4.1-4.6]	0.5
Chloride (mmol/L)	99.0	[97.5-101.5]	104.0	[102.5-104.5]	0.007
Osmolality (mOsm/kg)	282.0	[279.0-284.0]	287.0	[281.5-288.0]	0.09

Data are expressed as median [25th-75th percentile].

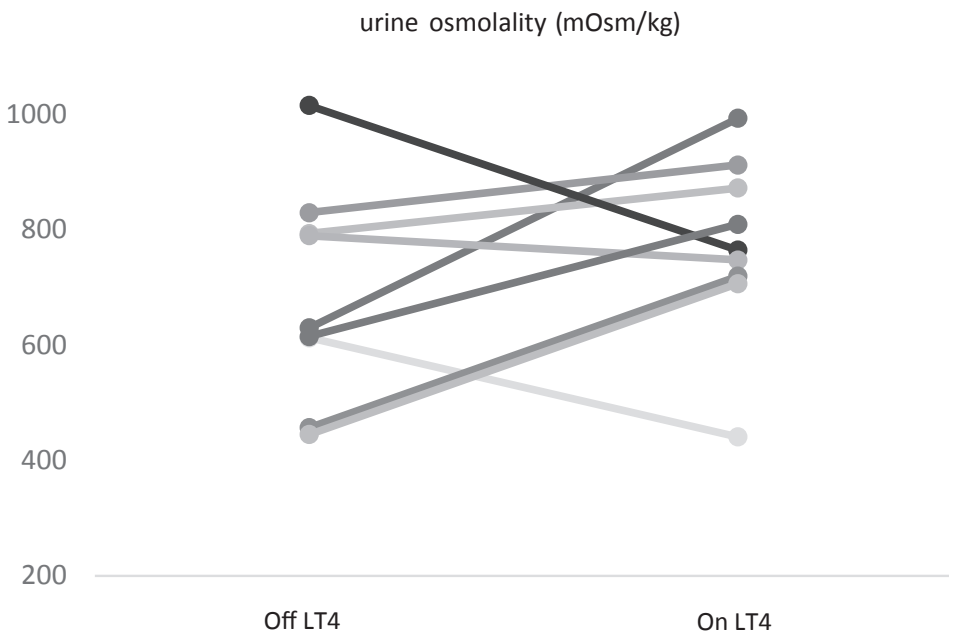

Figure 1. Changes in urine osmolality off and on LT4 treatment. Each line between two dots represents a patient.

Table 3. Changes in urine concentrations and osmolality

	Off LT4		LT4 treated		p-value
Osmolality (mOsm/kg)	630.0	[535.0-812.0]	765.0	[613.0-794.0]	0.17
Sodium (mmol/L)	84.0	[54.5-146.0]	141.0	[95.5-157.5]	0.21
Creatinine (mmol/L)	17.2	[12.3-28.5]	13.7	[12.1-20.6]	0.09
Sodium/ creatinine ratio	5.1	[1.9-10.8]	11.0	[5.1-12.0]	0.26
Urea (mmol/L)	258.0	[190.0-383.0]	328.0	[274.0-439.0]	0.09

Table 3 shows that urinary osmolality was not significantly different between patients on and off LT4 treatment (765.0 vs. 630.0 mOsm/kg, respectively; $p = 0.17$). Figure 1 shows the changes in urinary osmolality within each patient. There was also no significant difference in sodium, urea and creatinine levels in the urine samples. There was no correlation between urinary osmolality and age, TSH, FT4, total T4 and total T3 levels neither during hypothyroidism nor during LT4 treatment (data not shown).

Data are expressed as median [25th-75th percentile].

DISCUSSION

In this prospective study in athyreotic DTC patients there was neither a significant difference in urine osmolality nor in serum osmolality on and off LT4 treatment after a water and food deprivation test of 14 hours. Since we could not detect an impairment of urinary concentrating ability during severe hypothyroidism in our patients, we did not assess AVP and copeptin concentrations, a stable pre-pro-hormone of AVP. Our findings are in contrast with previously published studies in rats (4) and humans (5, 6). In these previous studies on urinary concentrating ability in humans, detailed thyroid function tests were not performed. The severity of hypothyroidism was predominantly based on clinical characteristics, which is not very precise (7). In the current study, we confirmed severe hypothyroidism biochemically with a median TSH level of 68 mU/L, and correspondingly low levels of FT4, total T4 and T3. Whereas previous studies were performed in patients with prolonged signs of hypothyroidism (i.e. myxedema), hypothyroidism in the current study existed only for four weeks. Although we cannot exclude that there would have been an impaired urinary concentrating ability after prolonged hypothyroidism, the current study excludes important acute consequences of altered thyroid hormone status.

Another speculative explanation for our findings could be that our patients were treated with relatively high dosages of LT4 to establish TSH suppression (median TSH concentration of 0.049 mU/L) because of their thyroid cancer. Although our patients were not overtly thyreotoxic, high TH levels are associated with a hyperdynamic circulation including increased cardiac

output and blood pressure and decreased systemic vascular peripheral resistance (8). These systemic hemodynamic alterations are known to be associated with increased renal hemodynamics and urine flow which might have decreased the urine osmolality in our LT4 treated patients (9). This mechanism is supported by Wang *et al*, who observed in hyperthyroid rats a significant increase in solute excretion in the presence of an AVP independent downregulation of aquaporin water channels (10). In healthy human subjects, water deprivation causes the plasma osmolality to rise above 280–290 mOsmol/kg, which leads to the release of AVP into the circulation. This results in increased water retention with a rise in urine osmolality to a maximum of 1000–1200 mOsmol/kg and restoration of plasma osmolality toward the reference range (11). Since the median urine osmolality was lower than 1000 mOsmol/kg in both thyroid states in our patients, one could speculate that there was a concentrating defect in both thyroid states and we were therefore not able to find a statistical significant difference in urine osmolality.

Serum creatinine levels were significantly higher in our patients during hypothyroidism than during LT4 treatment and, correspondingly, eGFR was significantly lower during hypothyroidism. This is in line with several case reports and case series (12-14). Previous detailed studies have shown that the changes in serum creatinine reflect actual changes in GFR instead of alterations in creatinine metabolism (15-17).

A limitation of our study is the small number of patients, which is a consequence of the steadily growing number of DTC patients that are treated with RAI after preparation with recombinant TSH instead of LT4 withdrawal (18). A second limitation is the low iodine diet which patients had to adhere to in order to increase the effectiveness of RAI treatment. Although this diet is different from a “low-sodium” diet, any foods containing iodized salt and sea salt were not allowed (19). Therefore, we cannot exclude that this diet might have influenced the sodium and chloride levels and hence osmolality in our patients during hypothyroidism. Indeed, the significantly lower serum sodium (without development of hyponatremia) and chloride levels and the decreased urinary sodium/creatinine ratio off LT4 therapy, thus during the low iodine diet, support this notion. Similar results have been reported by Vannucci *et al*, who also showed significantly lower serum sodium levels off LT4 prior to ablative RAI treatment without any correlation between serum TSH and sodium levels, suggesting that the reduction in sodium levels is unrelated to the hypothyroid status (20). Finally, our study had an outpatient design which precludes strict control of adherence to the water deprivation

protocol. However, in general, an expectedly adequate serum osmolality was observed, indicating adequate water deprivation.

In conclusion, although previous studies have shown an impaired urinary concentrating ability in patients with myxedema, we did not find any evidence for impaired urinary concentrating ability in patients with short-term but severe hypothyroidism.

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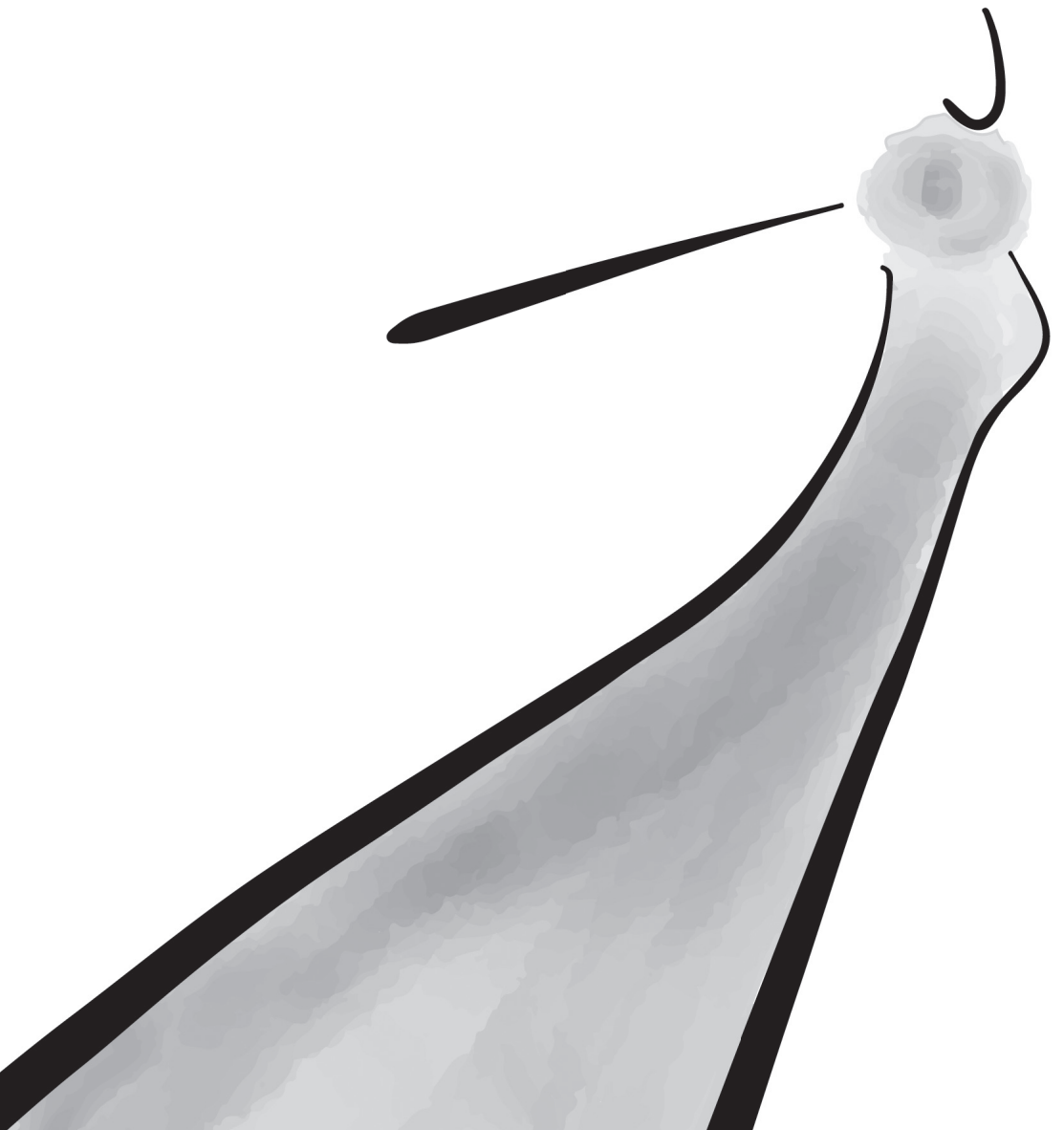

CHAPTER 6

Serum microRNA profiles in athyroid patients on and off levothyroxine therapy

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ABSTRACT

Background

Levothyroxine replacement treatment in hypothyroidism is unable to restore physiological thyroxine and triiodothyronine concentrations in serum and tissues completely. Normal serum thyroid stimulating hormone (TSH) concentrations reflect only pituitary euthyroidism and, therefore, novel biomarkers representing tissue-specific thyroid state are needed. MicroRNAs (miRNAs), small non-coding regulatory RNAs, exhibit tissue-specific expression patterns and can be detectable in serum. Previous studies have demonstrated differential expression of (precursors of) miRNAs in tissues under the influence of thyroid hormone.

Objective

To study if serum miRNA profiles are changed in different thyroid states.

Design and methods

We studied 13 athyroid patients (6 males) during TSH suppressive therapy and after 4 weeks of thyroid hormone withdrawal. A magnetic bead capture system was used to isolate 384 defined miRNAs from serum. Subsequently, the TaqMan Array Card 3.0 platform was used for profiling after individual target amplification.

Results

Mean age of the subjects was 44.0 years (range 20-61 years). Median TSH levels were 88.9 mU/L during levothyroxine withdrawal and 0.006 mU/L during LT4 treatment with a median dosage of 2.1 µg/kg. After normalization to allow inter-sample analysis, a paired analysis did not demonstrate a significant difference in expression of any of the 384 miRNAs analyzed on and off LT4 treatment.

Conclusion

Although we previously showed an up-regulation of pri-miRNAs 133b and 206 in hypothyroid state in skeletal muscle, the present study does not supply evidence that thyroid state also affects serum miRNAs in humans.

INTRODUCTION

Hypothyroidism is one of the most common endocrine disorders (1). In a subset (~10-15%) of patients, symptoms of hypothyroidism persist despite serum thyroid hormone concentrations within the laboratory reference range during levothyroxine (LT4) replacement therapy (2-4). One of the explanations for this impaired well-being might be the inadequacy of LT4 treatment to restore physiological thyroxine (T4) and triiodothyronine (T3) concentrations, especially the T4/T3 ratio, in serum and tissues (5, 6). Studies in hypothyroid rats showed that LT4 monotherapy was unable to normalize concentrations of T4 and T3 in all tissues (7). Supra-physiological serum T4 concentrations had to be reached in most tissues to normalize tissue T3 concentrations and the LT4 dose required to normalize thyroid hormone concentrations was different for each tissue.

Serum TSH concentrations, reflecting the pituitary feedback to thyroid hormone, is used in clinical practice to monitor LT4 treatment because it is the best available marker of thyroid state. However, although TSH typically reflects local T3 concentrations in the pituitary, it may not necessarily reflect local thyroid status in all tissues, especially when thyroid hormone production is not endogenously controlled, such as in athyreotic patients during LT4 therapy (8, 9). For this reason, novel markers representing thyroid state of other tissues than the pituitary would be of great clinical relevance.

MicroRNAs (miRNAs) are non-coding RNA molecules with a length of approximately 22 nucleotides, which predominantly post-transcriptionally repress the translation of miRNAs from target genes by binding to the 3'UTR of messenger RNA (10-12). Overall, miRNAs exhibit tissue-specific expression patterns and each miRNA may affect the expression of hundreds of target genes. Recently, we reported on gene expression profiles in skeletal muscle of hypothyroid patients off and on LT4 therapy and found a large up-regulation in expression of the muscle-specific pri-miRNAs 133b and 206 in hypothyroid state (13). This was supported by other studies that found an increase in expression of miRNAs- 1, 206, 133a and 133b in livers of hypothyroid mice compared to euthyroid controls (14). MiRNAs can also be present in the circulation and have been associated with a variety of diseases (15, 16). We therefore hypothesized that miRNA profiles in serum can also be influenced by thyroid state, and that miRNAs derived from different tissues potentially reflect tissue-specific differences in thyroid states.

MATERIALS AND METHODS

Patients

Patients with differentiated thyroid cancer (DTC) are exposed to different thyroid states as part of their treatment. These patients are subject to severe hypothyroidism before radioactive iodine (RAI) therapy to stimulate radioactive iodine uptake by malignant tissues, whereas they have relatively high thyroid hormone concentrations afterwards when receiving substitution therapy with LT4 to suppress TSH. As a consequence, these patients are an ideal model to study the consequences of different thyroid states longitudinally. Therefore, we used patients with DTC as a model to quantify serum levels of 384 miRNAs in different thyroid states. Since we were previously able to detect significant changes in numerous gene transcripts in peripheral blood as well as in muscle samples in respectively 8 and 10 patients, while using a similar study design, we postulated that 13 patients would be enough to identify clinically relevant markers (13, 17). Using alpha=0.05 and power=80%, we would be able to detect a Cohen's d effect size of 0.87, which is considered as a large effect.

13 DTC patients were consecutively recruited from the outpatient clinic of the Erasmus Medical Center, between May 1st 2013 and February 1st 2015. Patients were eligible for inclusion if they needed RAI therapy during LT4 withdrawal according to the Dutch guidelines (18), had no other malignancies or an active inflammatory disease, and were between 18 and 80 years old.

The study protocol was approved by the Medical Ethics Committee of the Erasmus Medical Center (MEC 2012-561) and written informed consent was obtained from all study participants.

Thyroid function measurements

Peripheral blood samples were obtained in non-fasting conditions from all participants on and off LT4 treatment. Serum Free T4 (FT4, reference range 11-25 pmol/L) and total T3 (reference range 1.4-2.5 nmol/L) concentrations were measured by chemoluminescence assays (Vitros ECI Immunodiagnostic System; Ortho-Clinical Diagnostics, Rochester, MI). Serum TSH concentration (reference range 0.4-4.3 mU/L) was determined by immunometric assay (Immulite 2000 XPi, Siemens, The Hague, the Netherlands). Serum samples were stored at −80°C until further analysis of miRNAs.

miRNA isolation from serum

The method of miRNA isolation and quantification has been previously described extensively (19). In short, the miRNAs were purified from serum

samples using TaqMan ABC Purification Kit – Human Panel A (Thermo Fisher, PN 4473087). These panels consist of superparamagnetic Dynabeads covalently bound to a unique set of 384 anti-miRNA oligonucleotides. The miRNAs match the miRNAs in Megaplex Pool described below. The panel includes exogenous and endogenous controls. Briefly, 100 µL of lysis buffer was added to 50 µL of serum. One µL of 1 nM of a non-human external control (ath-miRNA-159a) was added to monitor the extraction process, followed by the addition of 80 µL of beads (80×10^6 beads). The tubes were mixed for 40 min at 1200 rpm and 30 °C, the beads were isolated using a magnetic bead separator, and washed three times with wash buffer. The bound miRNAs were eluted from the beads with 100 µL elution buffer and incubated at 70 °C for 3 min. The eluted miRNA pool was stored at −80 °C until ready to use.

For miRNA profiling, Megaplex Primer Pool A was used in conjunction with the matching TaqMan miRNA Array Card A. All reagents were purchased from Thermo Fisher/Life Technologies (Bleiswijk, NL). Briefly, 3 µL of the miRNA sample isolated with the ABC kit was reverse transcribed with the Megaplex RT Primer Pool A (PN 4399966) in a 8 µL final volume. The RT reaction was performed under thermal cycling (2 min at 16 °C, 1 min at 42 °C, 1 sec at 50 °C, for 40 cycles) and the enzyme was inactivated by treatment for 5 min at 85 °C. Four µL RT reaction was combined with its matching Megaplex PreAmp Primer Pool (PN 4399233) and TaqMan PreAmp Master Mix (PN 4391128) in a final volume of 25 µL. Pre-amplification was done using the following cycling conditions: 10 min at 95 °C; 2 min at 55 °C; 2 min at 72 °C; 15 sec at 95 °C, 4 min at 60 °C for 12 cycles; 99 °C for 10 min. The final pre-amplification product was diluted 1:100 in 1X TaqMan Universal Master Mix (PN 4364341), then loaded onto the matching TaqMan MiRNA Array Card A (PN 4398965) and run on a TaqMan® 7900HT Fast Real-Time PCR System under universal cycling conditions.

miRNA QUANTIFICATION

Raw data files were imported and analyzed using ExpressionSuite v1.0.4 (Life Technologies, South San Francisco, CA, USA), a software data analysis tool that can easily import and analyze large raw data files. In these experiments, the quantification cycle (Cq) is defined as the fractional cycle at which the amplification plot crosses the fluorescence threshold (Ct). The baseline was set automatically and the threshold was manually set at 0.2 and adjusted whenever appropriate to get an intersection in the exponential part of the curve. To capture as many differentially expressed miRNAs as possible, the threshold was set at 40 instead of 30–32 which is generally recommended by

Life Technologies for relatively high miRNA levels. Undetermined values were replaced with the maximal number of cycles (=40) (20). The non-human external control (ath-miRNA 159a) differed between the serum samples with Cq values ranging between 23.0 and 26.9 (mean Cq value 24.4; SD 0.96). In previous studies using the same protocol, variation in spike-in controls has been reported to be similar (21).
TaqMan miRNA array output data (sds files) were uploaded in the ThermoFisher Cloud App (https://www.thermofisher.com/mysso/loginDisplay) and analyzed using defined threshold settings for each individual miRNA. Cq values were exported and filtered for poor amplification performance (Amplification Status, Amp Score and Cq Conf, Supplemental Table 1). MiRNAs with average Cq values below 37 were included for statistical analysis. In addition, miRNAs were selected which had Cq values<37 in all samples, representing abundant miRNAs.

Software and statistics

In order to correct for differences in input, global normalization was performed (Supplemental Table 1) using QbasePlus (Biogazelle N.V., Zwijnaarde, Belgium) according to Mestdagh *et al.*(22). A second method for normalization, also using endogenous controls, was performed. Based on an established algorithm for stability analysis (Normfinder), miRNA 93 and miRNA 130a turned out to be the most stable combination of miRNAs with mean Cq values (±SD) of 28.3 (±0.58) and 30.5 (±0.6) respectively (23). Normalization was also performed using the mean of these normalizers. Heatmaps of miRNA data were generated in R using the "pheatmap" clustering software package using the default settings. The Wilcoxon signed rank test was used for paired comparison of miRNA levels (ΔCq) and thyroid function tests on and off LT4 treatment. Multiple comparison adjustment was applied using the FDR approach (24).

RESULTS

Characteristics of 13 study patients are shown in Table 1. Mean age was 44.0 years (±SD 11.1) and mean BMI was 30.0 kg/m2 (±SD 6.0). Three of the patients (subjects 11-13) were treated with remnant ablation RAI therapy 4 weeks after thyroidectomy while the others were prepared for an extra RAI treatment by thyroid hormone withdrawal because of (suspicion of) residual or recurrent disease or positive anti-thyroglobulin (anti-Tg) antibodies.

Table 1. Baseline characteristics

Subject	Age (years)	Sex	BMI (kg/m^2)	Type of tumor	Comorbidity	Number of RAI treatments	Tg-off (µg/L)	Post-therapy ^{131}I scan
1	49	Male	25.8	PTC	Pulmonary embolism	1	3.9	No uptake
2	51	Female	34.1	PTC	none	1	2.1	No uptake
3	34	Male	36.4	PTC	none	4	517.0	Sacral metastasis
4	47	Female	32.9	PTC	hypoparathyroidism	1	94.4	No uptake
5	50	Male	24.5	PTC	none	3	5.6	LN
6	20	Female	20.7	PTC	hypoparathyroidism	1	<0.9*	No uptake
7	37	Female	24.6	PTC	none	2	21.8	Mediastinal LN
8	39	Male	40.5	PTC	none	3	12.9	No uptake
9	58	Female	29.0	FTC	none	1	<0.9	Not performed
10	61	Female	26.7	PTC	hypertension	1	21.6	No uptake
11	48	Female	35.1	PTC	hypertension	0	<0.9*	Thyroid remnant
12	45	Male	34.9	PTC	hypertension	0	7.5	Thyroid remnant
13	33	Male	25.0	PTC	none	0	<0.9*	Thyroid remnant

PTC, papillary thyroid carcinoma; FTC, follicular thyroid carcinoma; BMI, body mass index; RAI, radioactive iodine; Tg, thyroglobulin; LN, lymph node; *anti-Tg positive

As expected, thyroid function tests were significantly different on and off LT4 treatment (Table 2), reaching low levels of total T3 and FT4 with elevated TSH levels off LT4 replacement.

Table 2. Thyroid function tests

	Off LT4		On LT4		*p*
TSH (0.4-4.3 mU/L)	88.9	[56.5-118.5]	0.006	[0.004-0.015]	0.001
Total T3 (1.4-2.5 nmol/L)	0.64	[0.58-0.70]	2.13	[2.0-2.3]	0.001
Free T4 (11.0-25.0 *p*mol/L)	1.6	[0.4-1.8]	25.7	[22.4-29.3]	0.001
Dosage LT4 (µg/kg)			2.1	[1.9-2.6]	
Time between tests, weeks (range)			24.7	[11.0-38.8]	

Changes in thyroid function tests (normal range) off and on LT4 treatment. Data are presented as median with interquartile range. LT4, levothyroxine; TSH, thyroid stimulating hormone; T3, triiodothyronine.

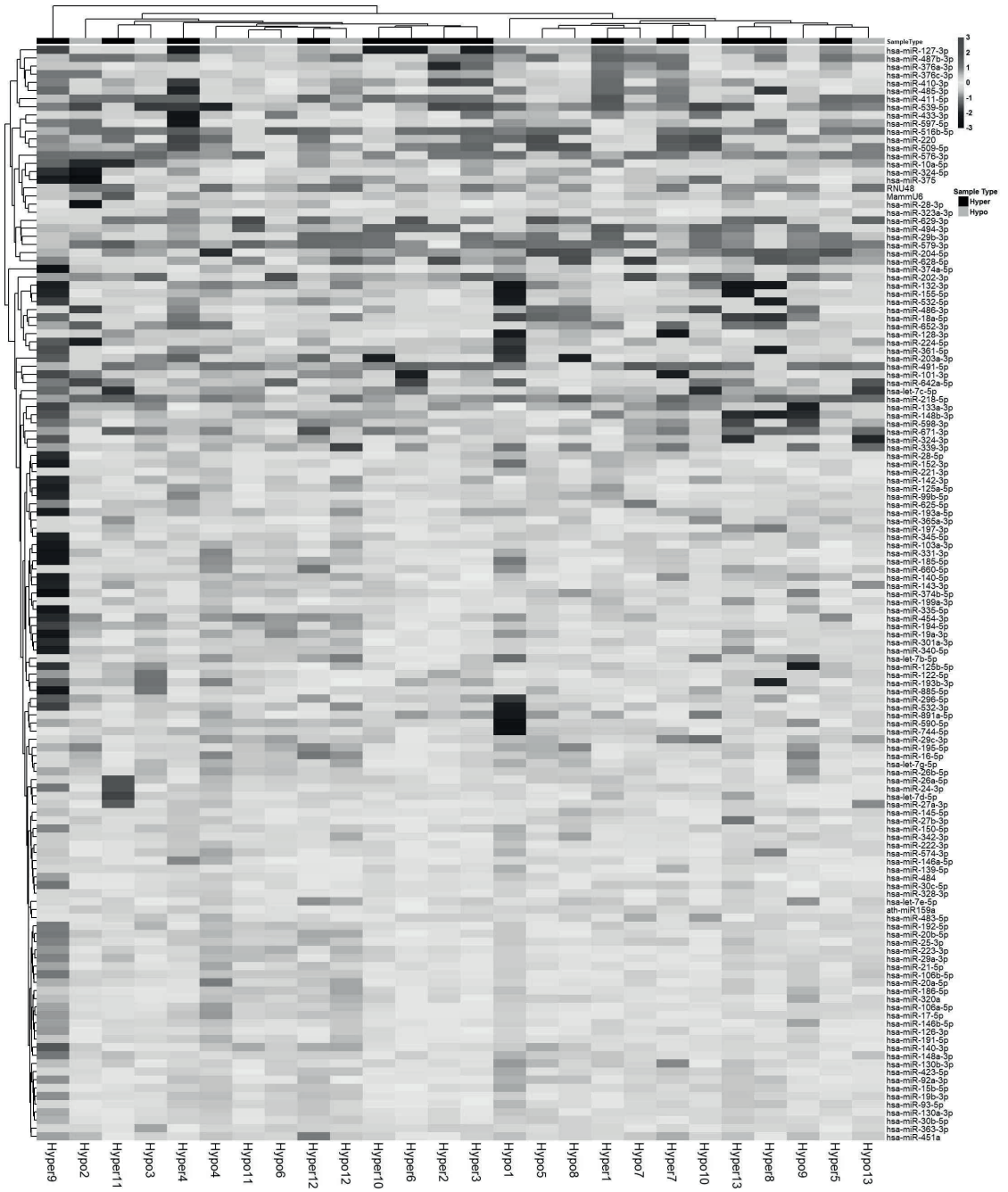


Figure 1. Heatmap showing the Cq values of all detectable miRNAs (n=135) in the different thyroid states. Clustering did not group the samples according to thyroid state.

Profiling of miRNAs

Out of the 384 miRNAs analyzed, almost half showed very low to undetectable levels. After removing all miRNAs with average Cq values > 37, only 135 miRNAs remained for analysis (Supplemental Table 1). Clustering of these globally normalized miRNAs did not group the samples according their thyroid state (Figure 1). The clustering of the abundant miRNAs (Cq values <37 in all samples, n=59) did not separate the samples according their thyroid state as well (Supplemental Figure 1). After calibration and normalization on the global mean, the statistical analysis revealed that none of the miRNAs was significantly altered between different thyroid states after correction for multiple testing (S1 Table). As a complementary approach, we normalized the dataset on the mean levels of two endogenous reference miRNAs (miRNA 93 and miRNA 130a). This confirmed the absence of any significant differences in levels of any of the miRNAs analyzed between thyroid states (Supplemental Table 1).

Previously reported miRNAs

The profiles of miRNAs which have been previously reported to be differentially expressed in muscle and liver in hypothyroidism were evaluated (13, 14). The level of miRNA 1 and miRNA 133b were not amongst the 135 detectable miRNAs (S1 Table). MiRNA 206 was not on the card. MiRNA 133a showed a mean Cq value of 31.2 (±1.2) in hypothyroid state versus 31.3 (±1.7) in hyperthyroid state (not significantly different).

DISCUSSION

To our knowledge, this is the first study investigating a selected multi-target miRNA profile in serum of individual patients during (extreme) changes in thyroid states. Most studies on miRNAs in patients with thyroid disease have focused on thyroid cancer (25). A study by Yamada *et al*, reported several miRNA levels that were differently expressed in serum from patients with autoimmune thyroid disease compared with healthy subjects (26). They suggested that the underlying autoimmune condition was responsible for the observed changes since there was no association between the mentioned miRNAs and TSH levels. However they did not study the changes in miRNA expression after restoring euthyroidism (26). Another study demonstrated that different levels of circulating miRNAs are associated with intractable Graves' disease compared with Graves' disease in remission (27).

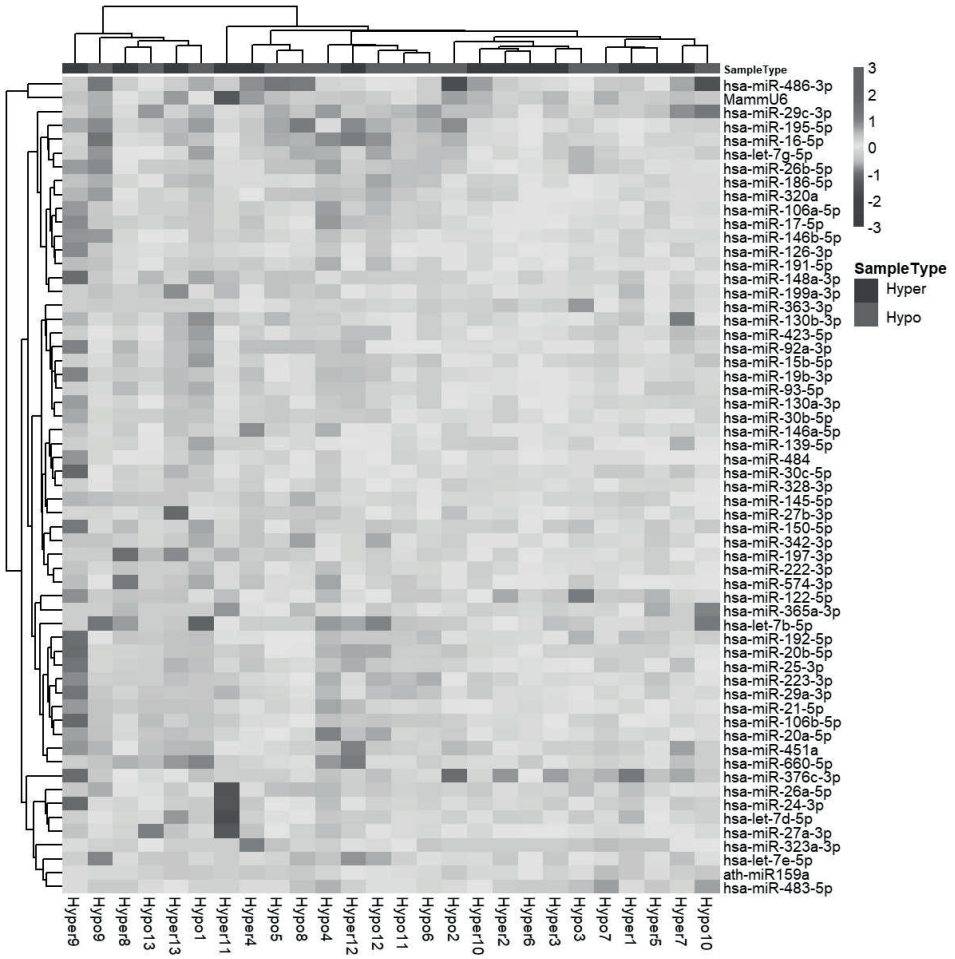
In our study, we could not find significant changes in levels of serum miRNAs in athyroid hypothyroid patients on and off LT4 treatment, despite previous studies showing clear differences in (precursors of) miRNAs in different tissues (13, 14, 28). There are a number of possible explanations for our results. First, our sample size might have been too small to discover significant changes in miRNA levels. However, previously, in a cohort of similar size, microarray analysis revealed large differences between thyroid states in muscle samples, suggesting that the used sample size was sufficient if the differences would have been equally large and consistent (13). McDonald *et al.* found that assay imprecision (due to variability in the RNA extraction process and interassay imprecision) had significant effects on the reproducibility of miRNA measurements and concluded that only miRNAs that are extremely up- or downregulated will be suitable as clinical biomarkers (29). If there were such extremely up- or downregulated miRNAs they should have been detected in our sample size. Second, although it has been shown that miRNAs are present in human plasma and serum in a remarkably stable form that is protected from endogenous RNase activity (15), differences in the time between blood drawing and storage potentially impact on the miRNA levels released from blood cells, in particular if samples are stored over 24h (29). However, all our samples were stored at -80 °C within 2 to 4 hours of collection and therefore a significant effect on miRNA expression is not likely (30). Third, although after calibration, normalization of the data was performed using two different methods (based on the global mean and based on mean levels of two endogenous reference miRNAs) providing similar results, we cannot rule out that a variation in protocol efficiency might have influenced our results. Finally, although several studies have shown that thyroid hormones seem to regulate miRNA expression in several organs such as skeletal muscle, liver and heart, these changes in tissue miRNA expression may not be reflected in serum (13, 14, 28). It has to be mentioned that our previous study in muscle samples examined only precursors of mature miRNAs (pri-miRNAs) while our current study investigated the effects of TH on the levels of mature miRNAs. However, Dong *et al.* found significant decreases of both mature miRNAs and it's corresponding precursor miRNAs in liver samples of hypothyroid mice compared to euthyroid controls (14).

Our study has several strengths and limitations. A limitation of our study is that we only studied a selection of 384 miRNAs and we cannot rule out that other miRNAs, which were not included in our platform are influenced by thyroid state. For example, miRNA 206, of which the precursor pri-miRNA-206 differed significantly in skeletal muscle, was not included in the current

platform. A possible confounder of our study is that we studied patients with DTC, while several miRNAs in serum have been postulated as biomarker for DTC (25). The levels of serum let-7e, miRNA-151-5p, microRNA-146b and miRNA-222 have been reported to be significantly increased in PTC patients relative to healthy controls (31, 32). Circulating levels of miRNA-146a-5p, miRNA-146b-5p, miRNA-221-3p, and miRNA-222-3p have been shown to decline after tumor excision (33). Although seven of our patients did not show any uptake on the post-therapy RAI scan and Tg-off levels were mostly low, some miRNAs theoretically could have been changed by the response of the tumor to RAI therapy or to the radiation it selves. Finally, although all patients with active inflammatory disease or other malignancies were excluded, some patients had comorbidities which might have affected their T3-dependent miRNA profile. Our study has several strengths as well. First, the study design included paired analyses, which has the advantage to reduce confounders. Second, we were able to study extreme differences in thyroid state in human subjects. Finally, we used a validated and widely used method to generate the serum miRNA profile (20, 34-36).

In conclusion, although we previously showed regulation of pri-miRNAs by thyroid hormone in muscle samples, the present study using an extensive panel of 384 miRNAs does not supply evidence of regulation of mature miRNAs in serum by thyroid hormone.

Supplemental Table 1. Raw data and results of the statistical analysis with normalization on the global mean.
https://doi.org/10.1371/journal.pone.0194259.s001



Supplemental Figure 1. Heatmap showing the Cq values of the abundant miRNAs (Cq values <37 in all samples, n=59) in the different thyroid states. Clustering did not group the samples according to thyroid state.

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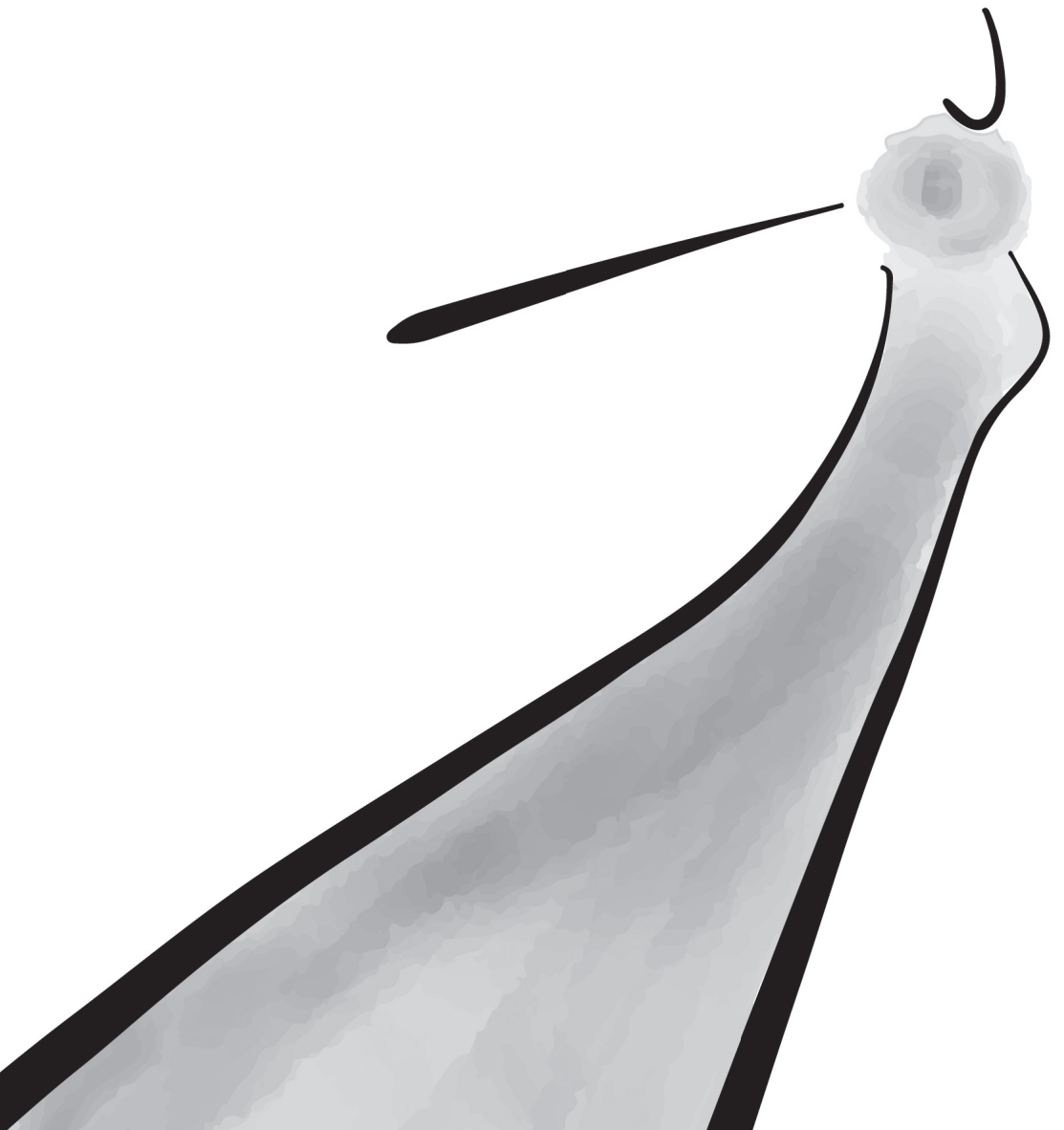

CHAPTER 7

Peptide Receptor Radionuclide Therapy in patients with Medullary Thyroid Carcinoma: predictors and pitfalls

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Submitted

ABSTRACT

Background

For progressive metastatic medullary thyroid carcinoma (MTC), the available treatment options with tyrosine kinase inhibitors result in grade 3-4 adverse events in a large number of patients. Peptide Receptor Radionuclide Therapy (PRRT), which has also been suggested to be a useful treatment for MTC is usually well tolerated, but evidence on its effectivity is very limited.

Objective

To evaluate the effectiveness of PRRT with ^{177}Lu-octreotate for MTC.

Methods

Retrospective evaluation of treatment effects of PRRT in a highly selected group of MTC patients, with progressive disease or refractory symptoms. In addition, a retrospective evaluation of uptake on historical ^{111}In-DTPA-octreotide scans was performed in patients with detectable tumor size >1cm.

Results

Over the last 17 years, 10 MTC patients were treated with PRRT. Four out of 10 patients showed stable disease at first follow-up (8 months after start of therapy) whereas the other 6 were progressive. Patients with stable disease were characterized by a combination of both a high uptake on ^{111}In-DTPA-octreotide scan (uptake grade ≥3) and a somatostatin receptor type 2a (SSTR2a) expression of the tumor by immunohistochemistry. Retrospective evaluation of historical ^{111}In-DTPA-octreotide scans of 35 non-treated MTC patients revealed low uptake (uptake grade 1) in the vast majority of patients 31/35 (89%) with intermediate uptake (uptake grade 2) in the remaining 4/35 (11%).

Conclusions

PRRT using ^{177}Lu-octreotate could be considered as a treatment in those patients with high uptake on ^{111}In-DTPA-octreotide scan (uptake grade 3) and SSTR2a expression in tumor histology. Since this high uptake was present in a very limited number of patients, this treatment is only suitable in a selected group of MTC patients.

Abbreviations

Calcitonin, CT; CEA, carcinoembryonic antigen; CI, confidence interval; GEP-NETs, gastroenteropancreatic neuroendocrine tumors; HR, hazard ratio; uptake grade 1= uptake < normal liver uptake; uptake grade 2= uptake equal to normal liver uptake; uptake grade 3= uptake > normal liver uptake; uptake grade 4= uptake > normal spleen or kidneys uptake; MTC, medullary thyroid carcinoma; OS, Overall Survival; P-NETs, pancreatic neuroendocrine tumors; PFS, progression-free survival; PR, partial response; PRRT, peptide receptor radionuclide therapy; RECIST, Response Evaluation Criteria In Solid Tumors; RET, rearranged during transfection; SSTR, somatostatin receptor; TKI, Tyrosine kinase inhibitor; WHO, world health organization

INTRODUCTION

Patients and treatment

Medullary thyroid carcinoma (MTC), originating from calcitonin (CT)-producing parafollicular C cells, is a rare form of thyroid cancer that accounts for less than 5% of thyroid carcinomas (1). In 25% of the cases, MTC is part of inherited disorders, such as multiple endocrine neoplasia 2a, 2b or familial MTC involving RET germline mutations. Locally unresectable tumor or distant metastases have limited systemic treatment options (2, 3). Although the tyrosine kinase inhibitors (TKI) vandetanib and cabozantinib have been shown to improve progression-free survival (PFS) [hazard ratio (HR), 0.46 and HR 0.28 respectively], grade 3 or 4 adverse events occur in a large number of patients (44% in vandetanib, 69% in cabozantinib) (3, 4). Therefore, alternative systemic treatment options with less side effects are needed.

Somatostatin receptor (SSTR) expression has been reported in up to 85% of MTCs, particularly SSTR subtypes 2, 3 and 5 (5-7), with 49% of MTCs showing expression of the SSTR2a subtype (5). Somatostatin receptor scintigraphy with ^{111}In-DTPA-octreotide (Octreoscan™), which has high affinity for SSTR2a, has been reported to show lesional uptake in 57-65% of MTC patients (8, 9). Therefore, targeting the tumor with a radionuclide using somatostatin analogs as a ligand seems to be an attractive option.

In midgut neuroendocrine tumors, peptide receptor radionuclide therapy (PRRT) resulted in a PFS rate at 20 months of 65% vs. 11% in the control group (10). In this trial, baseline ^{111}In-DTPA-octreotide uptake was positively correlated with remission rate (10).
In MTC there is limited experience with PRRT treatment. A phase II trial in 31 patients with ^{90}Y-DOTATOC, which also targets SSSTR2a, reported a partial response (PR) in 29% of the patients (11). In a second trial treating 7 MTC patients with ^{177}Lu-octreotate, 3 patients had PR, 3 patients had stable disease (SD) and 1 patient progressive disease (PD) (12). These results suggest that PRRT might be a useful treatment in patients with MTC, although the total number of treated patients is very limited so far. For that reason, we performed a retrospective evaluation of treatment with ^{177}Lu-octreotate in our center, where it was used in a highly selected group of 10 MTC patients with progressive disease or refractory symptoms. In addition, we evaluated possible predictors and pitfalls of ^{177}Lu-octreotate treatment in MTC.

METHODS

We retrospectively studied 10 consecutive patients with histologically proven MTC. Patients treated with ^{177}Lu-octreotate between 2000 and 2017 had progressive metastatic MTC according to Response Evaluation Criteria In Solid Tumors 1.1 (13) (RECIST) or had clinically refractory disease. The study was approved by the Institutional Review Board of the Erasmus Medical Center and written informed consent was obtained from participants.
We used the methods for ^{177}Lu-octreotate therapy as described in detail previously (14). Patients received an average of 4 cycles of ^{177}Lu-octreotate, up to a cumulative dose of 750 to 800 mCi, with an interval of 6 to 10 weeks (15, 16). Response to treatment was evaluated at a median of 8 months (3 months after the last cycle with ^{177}Lu-octreotate) and subsequently at 3 monthly follow-up visits, assessing clinical, biochemical and imaging parameters. The world health organization (WHO) performance status was scored at baseline and during follow-up by the treating physician.

End points

PFS was computed as the time from treatment initiation to progression, assessed by objective tumor response RECIST 1.1 criteria, clinical disease progression according to the treating physician, death or until the last date of follow-up (13). Overall survival (OS) was computed as the time from treatment initiation to death, or until the last date of follow-up. Adverse events were scored according to Common Terminology Criteria for Adverse Events (17).

^{111}In-DTPA-octreotide scans

We retrospectively reviewed historical ^{111}In-DTPA-octreotide scans between 1999 and 2011 of non-treated MTC patients that were performed in metastatic MTC. Although not part of the regular follow-up of MTC (2), ^{111}In-DTPA-octreotide scans were performed for tumor staging in most of these patients. Uptake was scored according to the Krenning score *(16, 18)*: uptake grade 1= uptake < normal liver uptake; uptake grade 2= uptake equal to normal liver uptake; uptake grade 3= uptake > normal liver uptake; uptake grade 4= uptake > normal spleen or kidneys uptake (Figure 1).

Immunohistochemistry SSTR2a

SSTR2a immunohistochemistry was retrospectively performed in the patients treated with ^{177}Lu-octreotate. Formalin fixed paraffin embedded (FFPE)

tissue slices of one or two tissue biopsies per patient were immunostained for SSTR2a using the Ventana BenchMark ULTRA stainer (Ventana, Tucson, Arizona, USA), according to the protocol provided by the manufacturer. The rabbit monoclonal anti-sst2 antibody (BioTrend, Köln, Germany) was used at a dilution of 1:25. Normal pancreatic tissue served as a positive control. SSTR2a expression was scored according to the percentage of cells with positive immunohistochemistry (0: absent staining; 1: weak staining <30% of cells; 2: moderate staining 30-60% cells; 3: strong staining >60% of cells). Scoring was done by two independent pathologists (F.H. N and M.F.V), who were blinded to each other's findings and patient data.

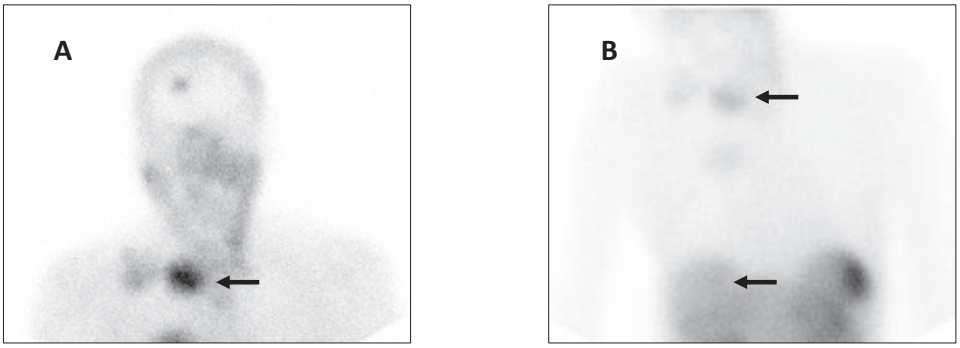


Figure 1. The principle of Krenning uptake on ^{111}In-DTPA-octreotide scans. A) MTC in neck region seems to have significant uptake. B) However when compared to normal liver uptake it is scored as Grade 2 uptake.

Laboratory measurements

Calcitonin (CT) and carcinoembryonic antigen (CEA) are useful tumor markers of residual disease and their doubling time is an indicator for prognosis (19). CT was measured on an Immulite 2000XPi immunoassay system (Siemens Healthcare Diagnostics, Los Angeles, USA) by chemoluminescence method. CEA was measured by an electrochemiluminescence immunoassay on a Cobas e601 immunoassay analyzer (Roche Diagnostics GmbH, Penzberg, Germany). The inter-day coefficient of variation of CT and CEA were 7.8% and 5%, respectively.

Statistics

We refrained from statistics due to limited patient number. PFS and OS was determined with Kaplan-Meier.

RESULTS

Baseline

Ten patients were treated with ^{177}Lu-octreotate. They had a median age 62 [range 19-75] years and 4/10 (40%) were male (Table 1). The indication for ^{177}Lu-octreotate was progressive MTC according to RECIST-criteria in 8/10 (80%) of patients. One patient was treated for intracardial metastasis with biochemical progression and one patient for large cervical inoperable tumor load. None of the tested patients showed germline mutations in the RET proto-oncogene (Table 1). Eight out of 10 (80%) patients had a baseline WHO performance status of 1, whereas the other 2 treated patients had a WHO status of 3 (Table 1). Three out of 10 (30%) patients had an endocrine paraneoplastic syndrome due to tumor-produced adrenocorticotropic hormone (ACTH), resulting in ectopic Cushing's disease, parathyroid hormone-related protein (PTH-rp) resulting in severe hypercalcemia and dopamine without clinical sequelae.

End points

Two patients showed PD during the third treatment cycle and were withheld from further treatment. In total, median PFS was 0.70 years [range 0.3-12.0], 1 patient is still in follow-up with stable disease 1.6 years after start of treatment. In total, 6 out of 10 (60%) patients had tumor progression at first follow-up (8 months) after start of treatment. Four out of 10 (40%) patients had SD at first follow-up. This includes, the patient with intracardial tumor mass with SD at start of therapy. Overall, SD was maintained for a median of 1.4 years [range 0.7-12.0]. One patient had enduring ustained SD for 12 years and died from an unrelated disease (fibrosarcoma).

Overall median OS was 1.14 years [range 0.4-12.0]. Two patients are still alive at 1.4 and 1.6 years since start of PRRT. The median OS in SD patients was 1.8 years [0.8-12.0].

Baseline CT doubling time and CEA doubling time were not associated with SD or PD outcome at 8 months (Table 1). None of the patients showed a decrease in CT or CEA levels of ≥ 50% after treatment with ^{177}Lu-octreotate. Three of the four patients with SD showed a 20-40% decrease of CT and/or CEA levels with sustained response. One out of these four patients showed an initial rise of tumor markers in the first 5 months, which was followed by a sustained decrease in both tumor markers. Four out of the 6 patients with PD showed loss of correlation with CT or CEA (no increase in tumor marker, despite progressive disease) (Table 2), which could be a pitfall in assessing response to therapy after PRRT.

Table 1. Patient characteristics

	Overall (N=10)	Stable disease (N=4)	Progressive disease (N=6)
Age years *median [range]*	63 [19-75]	69 [19-75]	60 [42-73]
Male *N (%)*	4/10 (40%)	1/4 (25%)	3/6 (50%)
RET			
Sporadic N (%)	6/10 (60%)	3/4 (75%)	3/6 (50%)
Unknown N (%)	4/10 (40%)	1/4 (25%)	3/6 (50%)
Disease extent			
Moderate	8/10 (80%)	2/4 (50%)	6/6 (100%)
Extensive	2/10 (20%)	2/4 (50%)	0/6 (0%)
Tumormarkers			
Calcitonin DT years, [range]	2.4 [0.6-4.1]	0.8 [0.6-4.1]	2.6 [2.4-2.9]
CEA DT years, [range]	1.9 [0.6-7.4]	1.9 [1.2-2.1]	4.0 [0.6-7.4]
PRRT indication			
PD	8/10 (70%)	3/4 (75%)	5/6 (83%)
Tumor localization/ refractory symptoms£	2/10 (30%)	1/4 (25%)	1/6 (17%)
WHO			
1	8/10 (80%)	3/4 (75%)	5/6 (83%)
3	2/10 (20%)	1/4 (25%)	1/6 (17%)
Hormonal functioning ¥	3/10 (30%)	1/4 (25%)	2/6 (33%)

CEA, carcinoembryonic antigen; DT, doubling time; N, number; PRRT, peptide receptor radionuclide therapy; RET, rearranged during transfection; WHO, world health organization performance status

£ Intracardial metastasis and biochemical progression; inoperable cervical tumor load
¥ ectopic ACTH; PTH-rp; dopamin

Table 2. Clinical characteristics and outcome

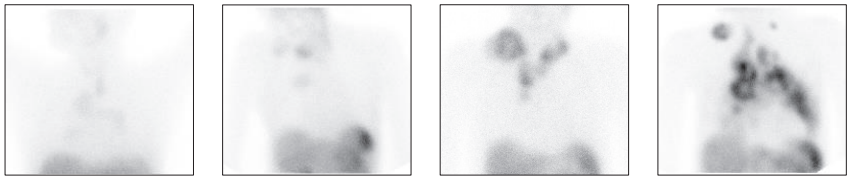
	Overall (N=10)	Stable disease (N=4)	Progressive disease (N=6)
Uptake Grade ≥3	7/10 (70%)	4/4 (100%)	3/6 (50%)
Uptake Grade <3	3/10 (30%)	0/0 (0%)	3/6 (50%)
SSTR2a tumor expression	4/10 (40%)	4/4 (100%)	0/6 (0%)
Loss of correlation CEA/CT	4/6 (67%)	0/4 (0%)	4/6 (66%)

Calcitonin, CT; CEA, carcinoembryonic antigen; Grade 3: uptake > normal liver uptake; N, number

Predictors of response

All 4 patients with SD showed high uptake on the ^{111}In-DTPA-octreotide scans (uptake grade ≥ 3) and showed moderate to positive SSTR2a receptor expression on histological examination (Table 2).

All patients with PD had negative tumor STTR2 expression in the tissue biopsy. Patients with PD had variable uptake on ^{111}In-DTPA-octreotide scans, as well as on post therapy scans (Table 2). Two out of 6 patients (33%) showed uptake grade 3 on ^{111}In-DTPA-octreotide scan. In one of these patients we identified a remarkable SSTR2a positive staining of tumor endothelium. In 1 ot of the 6 patients (17%) non-homogenous uptake of ^{111}In-DTPA-octreotide scan, with loss of uptake in some metastasis was observed. In the other patients, 1/6 (17%) showed uptake grade 2 and 2/6 (33%) showed uptake grade 1 (Figure 2).



Uptake	Grade 1	Grade 2	Grade 3	Grade 4
PRRT N(%)	2/10 (20%)	1/10 (10%)	3/10 (30%)	4/10 (40%)
SRS N(%)	31/35 (89%)	4/35 (11%)	0/35 (0%)	0/35 (0%)

Figure 2. Uptake on ^{111}In-DTPA-octreotide scans in patients treated with PRRT and of 35 non-treated patients with metastasized MTC.

^{111}In-DTPA-octreotide scans

None of the 35 retrospectively evaluated non-treated MTC patients had an ^{111}In-DTPA-octreotide scan with higher uptake in the metastases than in the liver (grade ≥3). Only 4/35 (11%) patients showed moderate uptake (grade 2). In the majority of patients 31/35 (89%), the tumor was detectable, but uptake on ^{111}In-DTPA-octreotide scan compared to liver uptake (Figure 2).

Symptoms and adverse events

There was no improvement of symptoms or paraneoplastic syndrome, neither in the stable disease patients nor in the progressive patients. Common grade 1 side effects included diarrhea, fatigue, mild anorexia and mild hair loss, occurring in the majority of patients. Grade 2 diarrhea occurred in 1 out

of 10 patients. One patient developed grade 3 adverse event: hemoptysis, presumably due to progression of pulmonary metastasis.

DISCUSSION

This study reports the results of 10 patients with metastasized MTC that were treated with PRRT using ^{177}Lu-octreotate. Only 4 patients showed SD at first follow-up at 8 months after start of therapy. All of these 4 patients with SD showed high uptake (Krenning uptake grade ≥3) on the ^{111}In-DTPA-octreotide scan as well as SSTR2a receptor expression in the tumor upon immunohistochemistry staining, whereas none of the remaining 6 patients that had PD at first follow-up had such a combination of high uptake plus SSTR2a receptor expression in the tumor. ^{177}Lu-octreotate was well tolerated, in accordance to previous publications (10).

Our results are similar to the results reported with ^{90}Yttrium-labelled octreotide treatment (11), where partial response (PR), defined as any decrease in tumor marker, was reported in 9 out of 31 (29%) of patients compared to 3 out of 10 (30%) of the patients in the present study. Moreover, median OS was in a similar range, i.e. 1.3 years in patients treated with ^{90}Yttrium-labelled octreotide vs. 1.14 years in our study population. However ,^{90}Yttrium-labelled octreotide is known to cause more severe hematological and renal side effects (11).
The trial with PRRT in seven MTC patients by Vaisman *et al.* reported 3/7 (42%) patients with PR, 3/7 (42%) patients with SD, and 1/7 (14%) with PD at 3 months follow-up (12). Although patient numbers are small, this suggests a better treatment response compared to the present study, where 6/10 (60%) patients had PD. PFS was 6.38 years in the study by Vasiman *et al.* versus 0.70 years in our patient population. This might indicate that our study population included patients with more aggressive MTC. Several other study characteristics support this hypothesis. Firstly, the present study included 3/10 (30%) patients with paraneoplastic endocrine syndrome, which is a well-known poor prognostic factor (20). Secondly 4/10 (40%) patients showed loss of tumor marker expression, which is also associated with worse prognosis (21). And finally, our patients were much older (median age of 62 years [range 19-75] in the current study versus 35 years [range 20-78] in the study by Vaisman *et al.*), which is relevant, as age is an important determinant of prognosis in MTC (22).

The hypothesis that our study population had a aggressive form of MTC is also supported by the very low PFS in the present study , 0.7 years compared to 1.6 years in the placebo group of the phase III trial of the ZETA study (vandetanib)) (4). Resuming, the above data suggest that patients treated with ^{177}Lu-octreotate in this retrospective study were highly selected and had aggressive MTC tumors.

Predictors of response

All 4 patients with SD were characterized by high on ^{111}In-DTPA-octreotide scan (grade ≥ 3) and SSTR2a tumor expression, which is in line with observations n GEP-NETs (15, 16).

In the previously mentioned trial with ^{90}Yttrium-labelled octreotide (12), tumor response did not correlate with ^{111}In-DTPA-octreotide uptake. In that study only 2 out of 9 (22%) of the responders had a high uptake (grade >3) vs. 6 out of 22 (27%) of the non-responders. However, response was based on tumor marker decrease and not scored according to RECIST criteria. SSTR2a staining on the tumor specimen was not performed.

Importantly, our retrospective evaluation of historical ^{111}In-DTPA-octreotide scans shows that only very few MTC patients have ^{111}In-DTPA-octreotide scans with uptake grade ≥3. According to current European Neuroendocrine Tumor Society guidelines, high uptake on ^{111}In-DTPA-octreotide scan is required to qualify for PRRT. Our data in MTC are in line with these diagnostic criteria for NET (23) and suggest that ^{177}Lu-octreotate may only be a suitable treatment option for a very limited group of patients (Figure 1). Other papers have reported uptake in up to 65% of MTC patients, but none of these studies reported a formal uptake grade with comparison to hepatic uptake (8, 9).

Surprisingly, we identified 3/6 (50%) patients in the PD group who had negative SSTR2a tumor staining upon immunohistochemistry, despite uptake grade ≥ 3 on ^{111}In-DTPA-octreotide scan. This might be explained by non-homogenous tumor expression of the SSTR2a receptor. Moreover, in one of these patients with PD, we identified SSTR2a immunohistochemistry uptake in the vascular endothelium instead of in the tumor itself, which may have resulted in uptake on ^{111}In-DTPA-octreotide scan. Although vascular tissue usually predominantly expresses SSTR1, the vascular SSRT2a expression in this tissue sample could explain this finding (24).

In the present study SSTR2a expression was absent in all 6 patients with PD, which suggests that lack of SSTR2a expression in the tissue biopsy may be a bad prognostic sign in patients with MTC. This is in line with pancreatic NET (P-NETs) and GEP-NETs, where low SSTR2a tumor expression has been shown to be associated with poor outcome and more aggressive grades of tumor (25-27). In a retrospective study in 97 patients with MTC, SSTR2a expression was significantly correlated with the presence of lymph node metastasis. However, prognosis was not investigated (28). In human MTC cell line cultures, SSTR2a activation inhibited tumor cell proliferation (6).

Limitations and future perspectives

A limitation of the present study is the small number of patients. Despite this, as very limited data of PRRT in MTC patients are currently available, the present study provides valuable insights about which patients might potentially benefit from PRRT. In addition, it clearly illustrates that this therapy may only be suitable for a selected group of patients. In the present study, ^{111}In-DTPA-octreotide scans were used to evaluate SSTR2a uptake in 35 non-treated patients, demonstrating very limited uptake in majority of patients. In the present study, 111In-DTPA-octreotide scans were used to evaluate SSTR2a uptake in 35 non-treated patients, demonstrating very limited uptake in majority of patients. The nowadays more commonly used 68Ga-DOTATATE PET scans have better imaging properties due to pharmacological (higher affinity to SSTR2a), technical (eg. positron imaging, attenuation correction), as well as physical (higher gamma energies) differences and is useful for more precise staging of the patient (29,30). 68Ga-DOTATATE PET to evaluate eligibility for PRRT might result in more patients to be treated. However, as we did not observe significant differences in the pretreatment uptake on the 111In-DTPA-octreotide scan and post-therapy scan following PRRT, the amount of absorbed radiation dose to the tumors might be less than what would be needed for adequate treatment.

Due to the low adverse events rate, ^{177}Lu-octreotate could be considered a potential therapy in patients with both high uptake on pretherapy ^{111}In-DTPA-octreotide scan and as well as SSTR2a tumor expression.

CONCLUSION

PRRT using ^{177}Lu-octreotate could be considered in MTC patients with both a high tumor uptake (≥grade 3) on ^{111}In-DTPA-octreotide scan as well as tumor SSTR2a receptor expression by immunohistochemistry. Further research is

needed to evaluate the effectiveness in these patients. Our retrospective data of 35 non-treated MTC patients, suggest that only minority of patients are eligible for ^{177}Lu-octreotate therapy.

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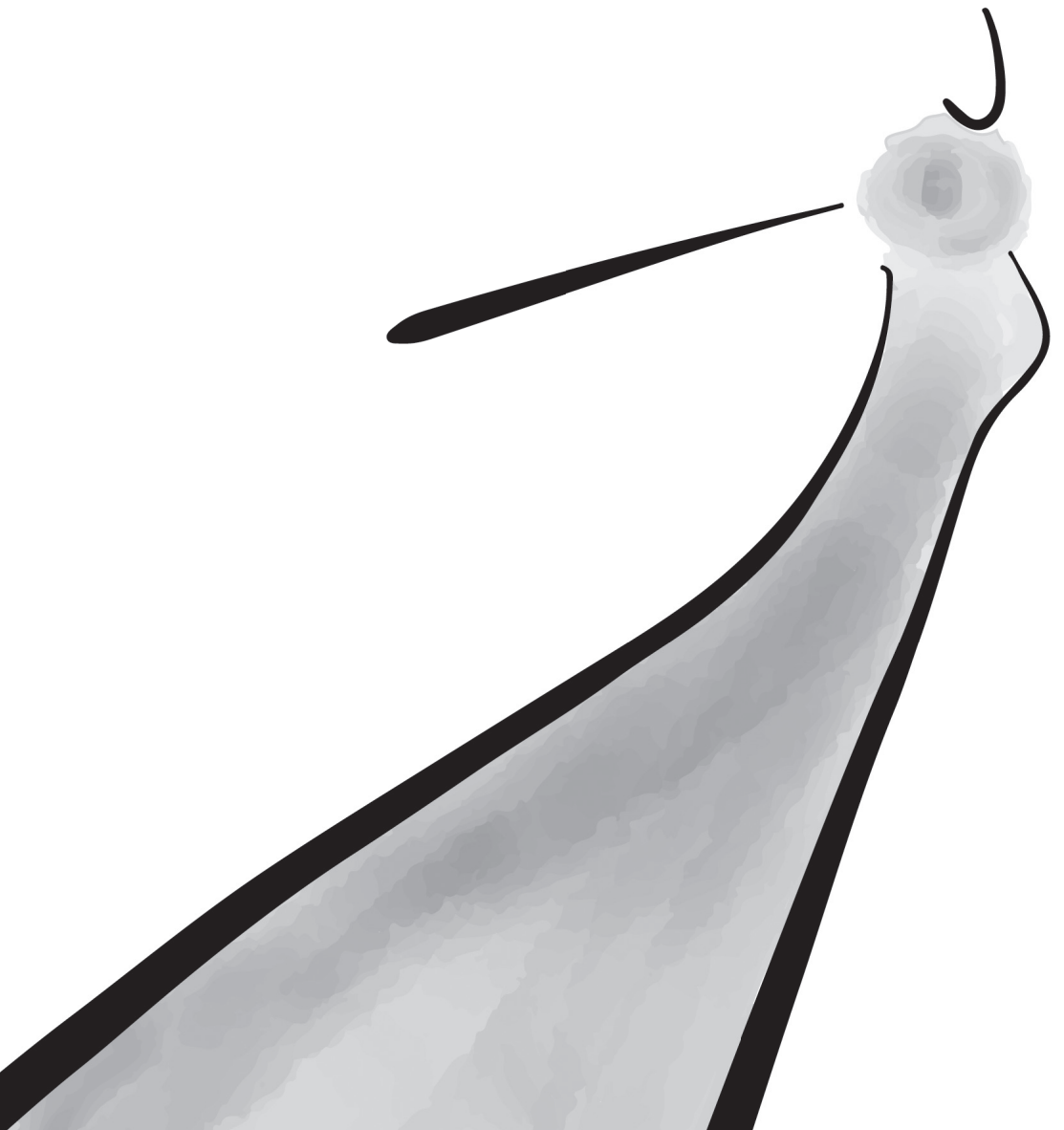


CHAPTER 8

Slipped capital femoral epiphysis as manifestation of a rare endocrinological disease

Beukhof CM, van Biezen FC, de Herder WW

Neth J Med. 2011;69(2):84, 94

CASE REPORT

Patient A presented at the age of 15 to the emergency department with acute hip complaints after a fall. At the age of six years, nodules were removed from her tongue. Examination revealed a flexion, abduction and exorotation contracture of the left hip with a decreased range of motion. Radiology confirmed a slipped capital femoral epiphysis (SCFE) of the left hip (Figure 1). The SCFE was surgically treated with cannulated screw fixation. Peroperatively the blood pressure remained stable. A few months later the patient was referred to an ENT specialist because of a nodule in the neck. Fine needle aspiration of the lymph node revealed medullary thyroid carcinoma. The patient had thickened lips, nodules on the tongue and a marfanoid appearance (Figure 2).

Patient B visited an orthopaedic surgeon at the age of 16 because of pain of the left hip on exertion for the last six months. On examination left hip flexion was diminished; however, abduction and adduction were within the normal range. Radiology of the left hip showed that the capital femoral epiphysis had slipped in a dorsomedial direction. Surgical treatment with a cannulated screw fixation was performed successfully. At the age of 17, the patient returned with an SCFE of the contralateral hip. Radiology confirmed a slip of the right epiphysis of the femoral head. No complications occurred during screw fixation. Preoperatively, a nodule in the thyroid had been noticed for which the patient was referred to a paediatrician. Thin needle biopsy showed no malignancy. Because of progressive growth of this tumour in the following years, which resulted in cosmetic complaints, the patient underwent a right hemi-thyroidectomy. Pathological examination surprisingly identified medullary thyroid carcinoma.

DIAGNOSIS

SCFE can be a manifestation of the multiple endocrine neoplasia syndrome type 2 (MEN 2) (1). MEN 2 syndrome is subdivided into MEN 2a and MEN 2b and both have medullary thyroid carcinoma as the most common feature. However, hyperparathyroidism is characteristic for MEN 2a whereas patients with MEN 2b can be recognised by neurofibromas of the tongue and marfanoid habitus (2). The diagnosis of acute SCFE is easier than that of chronic SCFE (3). Both disorders present with pain in the hip or with referred pain in the knee. Patients with acute SCFE typically have a contracture by flexion, abduction and exorotation. However, in patients with chronic SCFE the only

presenting symptom can be a mild limp (3). SCFE can be difficult to diagnose on anteroposterior radiographs (3). For chronic SCFE a lateral radiograph according to Lauenstein (hips in 90° flexion and maximal abduction) is advised (4). SCFE often occurs bilaterally, therefore bilateral imaging at presentation and also during follow-up is indicated (4) . Treatment of acute and chronic SCFE is surgical (4).

Awareness of the association between MEN 2 and SCFE could help to identify patients earlier. This is crucial in order to prevent metastatic medullary thyroid carcinoma. Pheochromocytoma can also be part of MEN 2 and could cause severe hypertensive crisis or arrhythmias perioperatively. Complications of SCFE are avascular necrosis, chondrolysis and coxarthrosis if the diagnosis is missed.

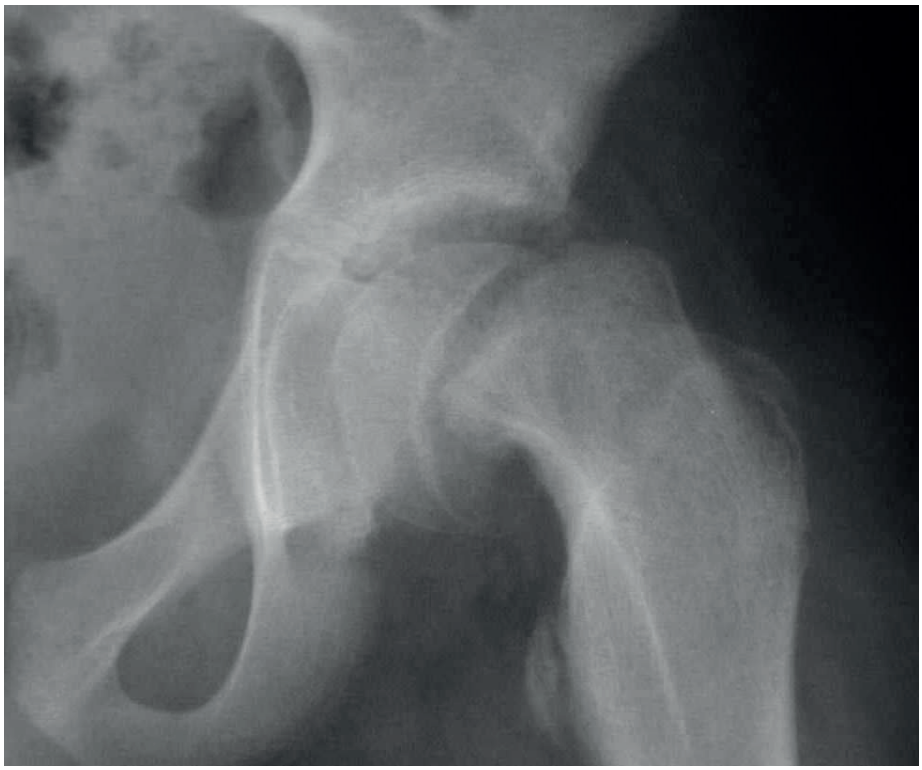
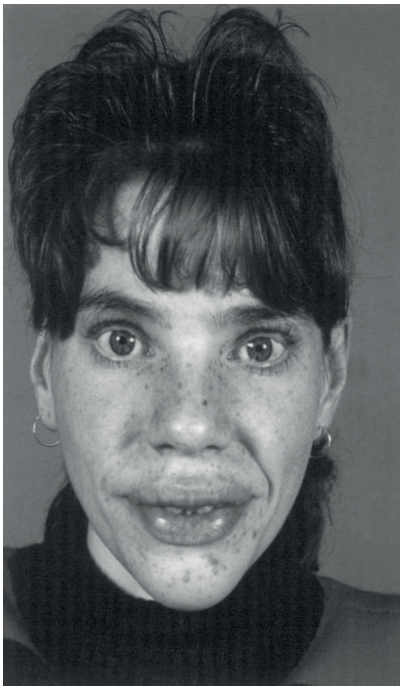


Figure 1. A typical example of an acute slipped capital femoral epiphysis.

a)



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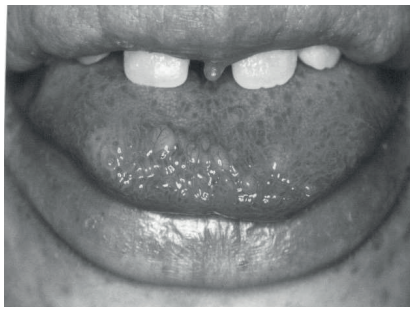

Figure 2. a) A patient with neurofibromas of the tongue and eyelids, thick lips and marfanoid phenotype. b) Close-up of neurofibromas of the tongue (permission granted)

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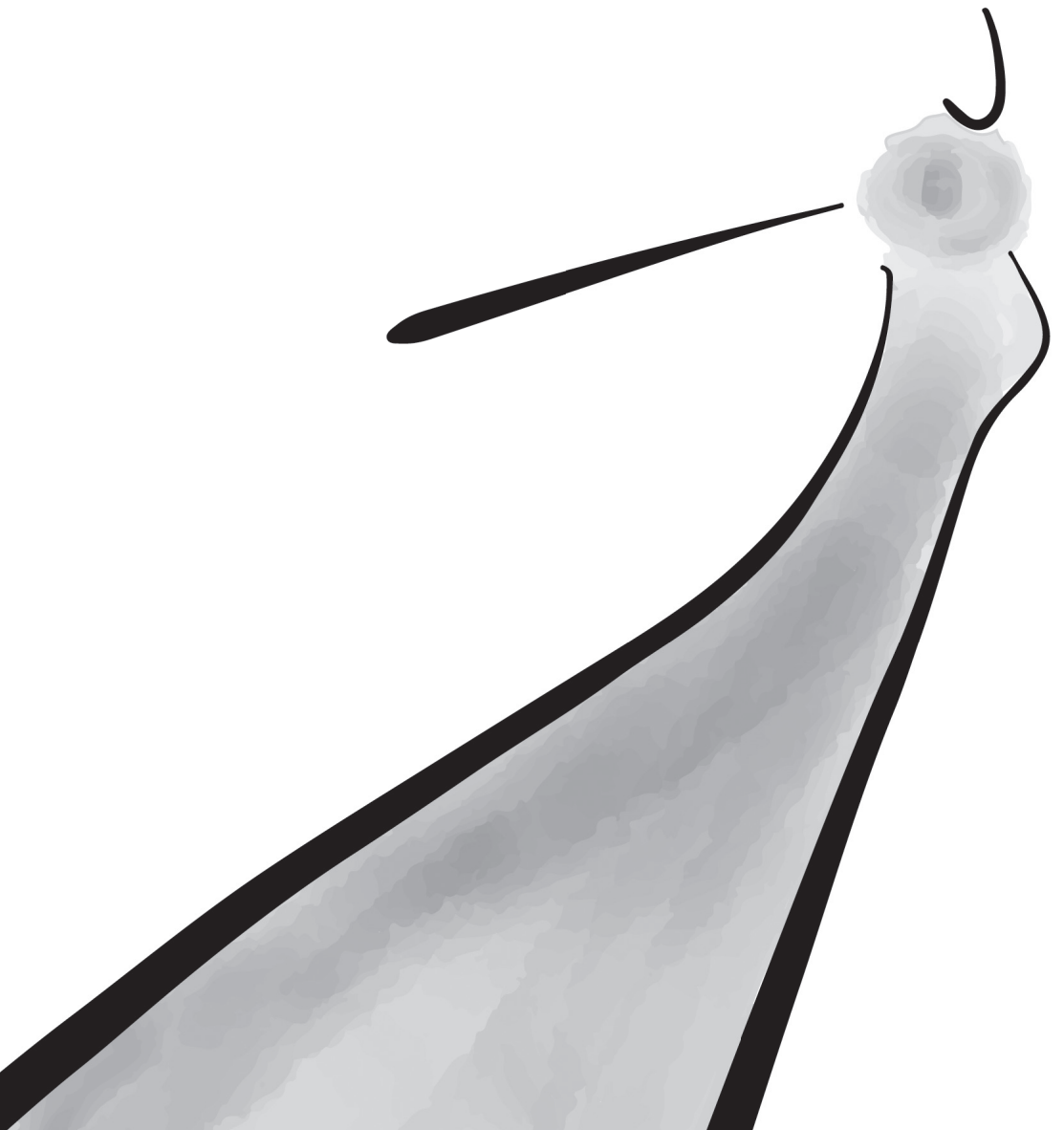

CHAPTER 9

Summarizing discussion and future perspectives

THYROID HORMONE

TH is essential for the metabolism in virtually all cells of the human body, especially the brain, heart, adipose tissue and muscles (1). In this thesis several clinical external factors such as dietary intake of Se, medication such as TKI as well as internal factors such as direct effects of TSH on TH metabolism and on TH action were investigated. In addition, the consequences of TH action on renal concentration capacity, possible biomarkers of tissue TH concentrations, and a novel treatment for MTC were studied.

Clinical factors affecting, thyroid hormone metabolism

External Factors

Nutritional trace element: Selenium

Many clinical factors are known to affect TH levels and metabolism. Dietary iodine deficiency, which is essential for normal TH synthesis, is still a concern in some developing countries (2). Se is another nutritional trace element that is essential for TH metabolism. TH action is regulated by the selenoenzymes D1-3, which all contain a selenocysteine in their catalytic centre (3). Extreme Se deficiency is associated with TH disturbances, Kashin-Beck osteoarthropathy and bone malformations (4). It has been reported that European populations' dietary Se intake is low-normal. However, it is still a matter of debate whether subtle changes in Se status are associated with alterations in TH concentrations (5). This is especially relevant in the elderly who are at risk for malnutrition (6). In **chapter 2**, the results of a cohort analysis in 387 elderly men aged ≥70 years (Zoetermeer study) are reported. The effects of Se status on TH concentrations were studied. TFT (T4, T3, rT3 and their ratios) were not affected by Se status in this population. Besides its role in TH metabolism, Se has many other actions including antioxidant defence and Se is associated with decreased inflammation in autoimmune disease (7). Se supplements significantly slowed down the progression of mild Graves' ophthalmopathy (8). Moreover, a positive association between Se status and BMD has been described in postmenopausal women (9). However, data on the association of Se status and BMD in elderly men are lacking (10-12). In **chapter 2,** the effects of Se status on BMD were evaluated. Se status was positively associated with BMD in healthy aging men. Although, women are known to be more susceptible to osteoporosis compared to men, but our current data demonstrate that Se status influences BMD in men as well. Interestingly, even in this population with borderline sufficient levels of Se,

there was a significant association between Se status and BMD independent of thyroid function. *In vitro,* the association between Se status and BMD could possibly be explained by the effects of Se on modulating oxidative stress and, secondary, osteoblast differentiation (13, 14). However, before establishing a causal relationship, the effect of Se supplementation on BMD and fractures should be investigated in a clinical trial in elderly patients.

Medication: tyrosine kinase inhibitor sorafenib

External factors, such as medication, can also affect TH metabolism. The effects of lithium, prednisone and amiodarone on TF are well known. Newer drugs, such as TKIs, have recently shown to be associated with changes in TFT as well (15). However, the underlying mechanisms are still largely unclear. **Chapter 3** describes a retrospective cohort study in 57 patients with HCC treated with the TKI sorafenib. Our study demonstrates that sorafenib likely affects TFTs via multiple mechanisms. Thyroiditis occurred in 7% of the patients. Among the other patients, 30% had a combined increase of TSH and FT4 above the normal range, suggesting central adaptation of the HPT-axis. In addition, there was a marked decrease in the T3/rT3 and T3/T4 ratios during treatment with sorafenib. This could be explained by an effect of sorafenib on peripheral TH metabolism. Finally, it was demonstrated that sorafenib decreased the cellular uptake of TH via MCT8 and 10 *in-vitro*. In addition, the chapter shows that the increase in TSH is an independent negative prognostic marker for PFS. Future studies are needed to unravel if and how the effects of sorafenib on thyroid function can be regarded as a prognostic factor and how changes in TFTs are best managed. TH disturbances are also reported in patients treated with other therapeutics, especially immune modulating antibodies, such as check point inhibitors (16) and interferon alpha (17). It is important during the development of such therapeutic agents, to closely monitor for thyroid disturbances and other endocrinopathies, e.g. pituitary and adrenal dysfunction. If these are identified, their etiology should be further investigated for adequate management.

Internal factors

Thyroid stimulating hormone

Besides external factors, TH metabolism is influenced by internal factors such as certain illnesses or pregnancy. However, it is not clear whether the internal factor TSH itself can have extra-thyroidal effects can change peripheral TH metabolism. Subclinical hypothyroidism is defined by an elevated TSH

level while T4 and T3 levels are within the reference range. This condition is associated with increased cardiovascular risk and elevated levels of total cholesterol and LDL-C (18-24). It is likely that this association is caused by slightly lower TH levels rather than by elevated TSH levels (25, 26). However, the presence of extra-thyroidal TSH receptors has been demonstrated in extraocular muscle, bone, fat tissue and liver, indicating that TSH may also have extra-thyroidal effects (27-29). In **chapter 4** the possible direct effects of TSH on peripheral TH metabolism and serum lipids were studied in 81 patients with DTC following successful radical thyroidectomy with subsequent ^{131}I ablation. During follow-up, thyroglobulin levels (indicating residual thyroid tissue which, is used as a tumor marker in these patients) were measured after 2 injections with rhTSH. In these patients we observed a significant decrease in T3 and FT3 from day 1 to day 5 despite a stable doses of LT4 supplementation. In addition, median Apo B, Lp(a) and triglyceride levels increased significantly, whereas serum HDL-C decreased. Multiple regression analysis showed that the changes in lipids were most closely associated with the decrease in T3 levels. Further studies with direct testing and studies in larger placebo-controlled cohorts, including subjects without DTC, are needed to confirm these findings. For future research we recommend that changes in TH metabolism are taken into account, before conclusions about direct effects of TSH can be drawn. Besides, it still needs to be investigated whether sustained elevation of TSH also causes an enduring change in TH metabolism as well as atherogenic alterations in serum lipids in patients with intact thyroid glands.

Consequences of thyroid hormone action

Effects on renal concentration ability

Data on the urinary concentrating defects in hypothyroidism in humans are limited (30, 31). The few studies that are available investigated healthy controls or were performed in patients with autoimmune thyroid disease. Furthermore, detailed TFTs were not performed (32). Therefore, in **chapter 5,** we investigated the effect of acute hypothyroidism on renal concentration ability in 9 athyreotic patients, on and off treatment with LT4 (i.e. during short-term severe hypothyroid state). Serum creatinine levels were significantly higher in patients off vs. on levothyroxine treatment. Correspondingly, the estimated glomerular filtration rates were significantly lower. This is in line with previous observations. No significant differences in serum and urine osmolality on and off levothyroxine treatment were found.

It cannot be excluded that the relatively high dosages of LT4 to attain TSH suppression in our patients have affected the results. Moreover, the effects on renal concentration ability in prolonged hypothyroidism might be different. However, the present study indicates that in states of acute hypothyroidism, for example following thyroidectomy in DTC patients, it is not necessary to take into account changes in urinary concentrating ability.

Effects on miRNAs

In clinical practice, LT4 substitution therapy is monitored by serum concentrations of TSH. Other markers, such as serum sex hormone binding globulin or creatine kinase, can be affected by TH, but these markers are not reliable in daily clinical practice. LT4 replacement therapy in hypothyroidism is generally insufficient to completely restore physiological T4 and T3 concentrations in serum and tissues (33). By definition, normal serum TSH concentrations only reflect pituitary euthyroidism and, therefore, novel biomarkers representing tissue-specific thyroid status of other tissues are needed. MiRNAs, small non-coding regulatory RNAs, exhibit tissue-specific expression patterns and are detectable in serum. Previous studies have demonstrated differential expression of (precursors of) miRNAs in tissues, influenced by TH. In **chapter 6**, 384 defined miRNAs were studied in 13 athyroid patients during TSH suppressive therapy and after 4 weeks of LT4 withdrawal. The present study, did not provide evidence that serum miRNAs are useful markers for hypothyroidism in humans. Although previous studies have shown that THs regulate miRNA expression in several organs, including skeletal muscle, liver and heart, these changes in tissue miRNA expression may not be reflected in serum (34-36). A limitation of this study is that we studied a selection of 384 miRNAs and it cannot be ruled out that other miRNAs, that were not included in this platform, are influenced by thyroid state. Further research is needed to find biomarkers that reflect tissue specific thyroid status.

Clinical factors affecting treatment outcome of thyroid diseases

Medullary thyroid carcinoma

For progressive MTC, the available treatment with TKIs, frequently causes grade 3-4 adverse events, resulting in discontinuation of treatment in 12-16% of patients, and additionally to dose reduction in 35-79% of patients (37, 38). Therefore, alternative systemic treatment options with less severe side effects are needed. **Chapter 7** is dedicated to PRRT with ^{177}Lu-octreotate

treatment for patients with MTC, which targets SSTR2a. In patients with inoperable metastatic midgut neuroendocrine tumors, PRRT with ^{177}Lu-octreotate, resulted in a significantly longer PFS rate at 20 months of 65.2% vs. 10.8% in the control group, who were treated with high doses of non-radiolabeled somatostatin analogs (39). PRRT is generally well tolerated (39). There is very limited experience with PRRT treatment for MTC patients. Therefore, a retrospective evaluation of treatment with ^{177}Lu-octreotate in MTC patients treated in our center was performed. PRRT with ^{177}Lu-octreotate was administered to 10 MTC patients with progressive disease or refractory symptoms. Our data indicate that PRRT for MTC could be considered as a treatment option in patients with both a high tumor uptake (≥ grade 3) on a ^{111}In-DTPA-octreotide scan as well as SSTR2a expression by tumor immunohistochemistry. However, our data derived from 35 ^{111}In-DTPA-octreotide scans performed in untreated patients with MTC suggest that only a minority of MTC patients are eligible for ^{177}Lu-octreotate therapy. Larger numbers of MTC patients have to be treated with PRRT, before definite conclusions about the effectiveness of this therapy can be drawn.

Strengths and weaknesses

The studies from this thesis were all conducted in a clinical setting. The etiology of frequently encountered clinical factors influencing TH action and metabolism were studied, while we also aimed to confirm these observations by direct testing *in-vitro*. We demonstrated, that a clinical model using paired analysis of patients on and off levothyroxine, and stimulation of rhTSH, provides new insights in TH action and metabolism. Our observations should be repeated in larger patient cohorts, in patients with an intact thyroid and in patients without suppressive TSH therapy. Besides, additional *in-vitro* experiments are necessary to further unravel the etiology of some of our observations.

CONCLUSIONS

Several clinical factors e.g. dietary intake of Se, medication including the TKI sorafenib, but also internal factors, such as TSH stimulation, can affect TH metabolism. It is important to account for all these factors in the treatment of patients. For the correct interpretation of thyroid function, we need biomarkers other than TSH. This thesis does not support the use of miRNAs as a valuable biomarkers in hypothyroidism. Finally, in the treatment of thyroid diseases there is a constant search for maximal effective therapy while

avoiding side effects. This thesis shows that PRRT using ^{177}Lu-octreotate can be useful in a highly selected subgroup of MTC patients with high uptake on ^{111}In-DTPA-octreotide scan as well as positive SSTR2a expression in tumor histology.

FUTURE PERSPECTIVES

This thesis shows the complexity of clinical factors affecting TH action and treatment outcome of thyroid diseases. Further research is needed to unravel pathophysiology, and to find new biomarkers and effective therapies that will be useful in day to day clinical practice.

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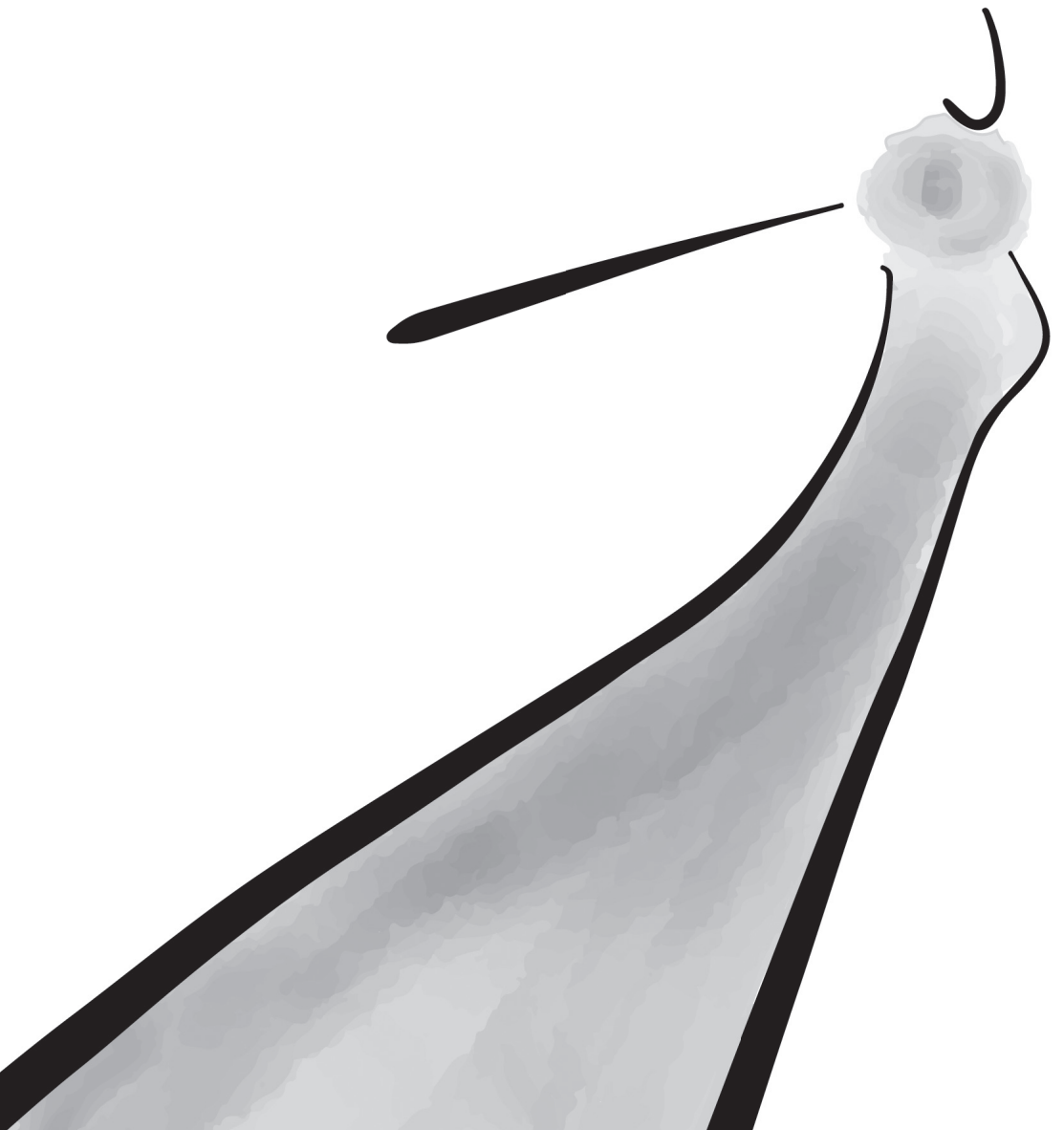

SUMMARY (ENGLISH)

Thyroid hormones are essential for normal functioning of the human body and almost all organ systems. Too little thyroid hormone (hypothyroidism) can result in fatigue, weight gain, cold intolerance, constipation, myopathy and also dyslipidemia and changes in renal function. Too much thyroid hormone secretion (hyperthyroidism) can result in weight loss, heat intolerance, frequent bowel movements and psychological changes.

Chapter 1 provides a brief background about regulation of thyroid hormone synthesis by the hypothalamic-pituitary-thyroid axis, thyroid hormone metabolism by deiodinases and thyroid hormone action via thyroid hormone receptors and transporters. The treatment of thyroid diseases, including hypothyroidism, hyperthyroidism and thyroid cancer, is described. The outline of this thesis "*clinical factors affecting thyroid hormone action and treatment outcome of thyroid diseases*" is discussed.

Clinical factors affecting thyroid hormone metabolism

External Factors

Nutritional trace element: selenium

Chapter 2 demonstrates that selenium status in a Dutch cohort of 387 healthy elderly men (at least 70 years of age), is within the normal European marginally supplied range and does not affect thyroid function tests. It is however positively associated with bone mineral density independent of thyroid function.

Medication: tyrosine kinase inhibitor sorafenib

Chapter 3 shows in a retrospective cohort study in 57 patients with hepatocellular carcinoma, that sorafenib likely affects thyroid function tests via several mechanisms. Thyroiditis occurred in 7% of the patients. In the other patients we found a combined increase of TSH and FT4, which suggests a central adaptation of the hypothalamic-pituitary-thyroid axis. A marked decrease in the T3/rT3 ratio and T3/T4 ratios during treatment with sorafenib could be explained by the effect on peripheral thyroid hormone metabolism. Sorafenib decreased cellular uptake of thyroid hormone via monocarboxylate transporter 8 and in lesser extent monocarboxylate transporter 10 *in-vitro*.

Internal Factors

Thyroid stimulating hormone

In **chapter 4** direct effects of TSH on peripheral thyroid hormone metabolism and serum lipids were studied in 82 patients with differentiated thyroid carcinoma who had undergone total thyroidectomy and radioactive Iodine remnant ablation. Two successive injections of recombinant human TSH were administered to patients on a stable dose of levothyroxine. This resulted in a significant decrease in serum T3 and FT3 concentrations. It was accompanied by a more atherogenic serum lipid profile with a significant increase in median apolipoprotein B, lipoprotein(a) and triglycerides, and a decrease in high density lipoprotein cholesterol. Multiple regression analysis showed that the changes in lipids were most closely associated with the decrease in serum T3 levels.

Consequences of thyroid hormone action

Effects on renal concentration ability

In **chapter 5** we analyzed the effect of acute hypothyroidism on renal concentration ability, in 9 athyreotic patients with differentiated thyroid carcinoma, during withdrawal of levothyroxine. Serum and urine osmolality were not significantly different between patients on or off levothyroxine treatment. Besides our study confirms the well-known effects of thyroid hormone on serum creatinine concentrations.

Effects on MicroRNAs

In **chapter 6** we searched for novel biomarkers representing thyroid state. We determined the effect of 4 weeks of levothyroxine withdrawal on microRNAs in the serum of 13 athyreotic patients with differentiated thyroid carcinoma prior to radioactive Iodine therapy. The present study shows that microRNAs are not affected by short term hypothyroidism and therefore does not support a role for microRNAs as biomarker for monitoring tissue-specific thyroid hormone status.

Clinical factors affecting treatment outcome of thyroid diseases

Medullary thyroid carcinoma

In **chapter 7** we retrospectively assessed the use of peptide receptor radionuclide therapy with ^{177}Lu-octreotate in 10 patients with medullary thyroid carcinoma with progressive disease or refractory symptoms. Our

data indicate that peptide receptor radionuclide therapy for medullary thyroid carcinoma could be considered as a treatment option in patients with both a high tumor uptake (≥ grade 3) on a ^{111}In-DTPA-octreotide scan as well as SSTR2a expression by tumor immunohistochemistry. However, our data derived from 35 ^{111}In-DTPA-octreotide scans performed in untreated patients with MTC suggest that only minority of MTC patients are eligible for ^{177}Lu-octreotate therapy.

SAMENVATTING (NEDERLANDS)

Schildkierhormonen zijn essentieel voor het functioneren van het lichaam en vrijwel alle organen. Te weinig schildklierhormoon (hypothyreoïdie) leidt tot vermoeidheid, gewichtstoename, koude intolerantie, obstipatie, spierpijn en ook dyslipidemie (verstoring van vetten in bloed) en verandering van de nierfunctie. Teveel schildklierhormoon (hyperthyreoïdie) kan resulteren in gewichtsverlies, warmte-intolerantie, diarree en psychologische veranderingen.

Hoofdstuk 1 geeft een korte achtergrond over regulatie van de schildklierhormoon balans door de hypothalamus-hypofyse-schildklier-as, schildklierhormoonmetabolisme door dejodases, en schildklierhormoonactie via schildklierhormoon receptoren en transporters. Introductie in de behandeling van schildklierziekten zoals hyperthyreoïdie, hypothyreoïdie en schildklierkanker wordt beschreven. De opzet van dit manuscript "*klinische factoren van invloed op schildklierhormoonactie en de behandeluitkomst van schildklierziekten*" wordt besproken.

Klinische factoren van invloed op schildklierhormoon-metabolisme

Externe factoren

Voeding spoorelement selenium:

Hoofdstuk 2 laat zien dat seleniumstatus binnen de bekende Europese laag normale waarde valt en dat dit de schildklierfunctietesten niet beïnvloedt in een Nederlands cohort van 387 gezonde oudere mannen (≥70 jaar). Seleniumstatus is wel positief geassocieerd met botdichtheid, onafhankelijk van de schildklierfunctie.

Medicatie: tyrosine kinase remmer sorafenib

Hoofdstuk 3 laat in een retrospectieve cohort studie in 57 patiënten met hepatocellulair carcinoom zien dat sorafenib schildkierfunctietesten via meer dan één mechanisme beïnvloedt. Thyreoïditis (schildklierontsteking) ontwikkelde zich in 7% van de patiënten. In de andere patiënten ontstond een gecombineerde verhoging van thyroïd stimulerend hormoon (TSH) en vrij schildklierhormoon (FT4), wat een centrale aanpassing van de hypothalamus-hypofyse-schildkier-as suggereert. Een uitgesproken verhoging in T3/rT3 ratio en T3/T4 ratio gedurende de behandeling met sorafenib zou verklaard kunnen worden door beïnvloeding van het perifere schildklierhormoonmetabolisme.

In normale niercellen remt sorafenib de intracellulaire opname van schildkierhormoon via de schildklierhormoon transporter monocarboxylaat transporter 8 en in mindere mate monocarboxylaat transporter 10.

Interne factoren

Thyroïd stimulerend hormoon

In **hoofdstuk 4** worden de directe effecten van TSH op het perifere schildklierhormoonmetabolisme en serum lipiden onderzocht in 82 patiënten met een gedifferentieerd schildkliercarcinoom die een thyroïdectomie (operatieve verwijdering schildklier) en radioactief jodium behandeling hebben ondergaan. Twee injecties met recombinant humaan TSH tijdens stabiele levothyroxine suppletie werden toegediend. Dit resulteerde in significante afname van serum T3 en FT3 concentraties. Daarnaast ontstond een meer atherogeen serum lipiden profiel, met een significantie toename van apolipoproteïne B, lipoproteïne(a) en triglyceriden, met een daling van hoge-dichtheid lipoproteïne cholesterol. Multipele regressie analyse laat zien dat de verandering in serum lipiden geassocieerd was met de daling van de T3 waardes.

Consequenties van schildkierhormoonactie

Effecten op renale urineconcentratie

In **hoofdstuk 5** werden de effecten van acute hypothyreoïdie op renale urineconcentratie bestudeerd in 9 patiënten zonder schildklier (status na gedifferentieerd schildkliercarcinoom) tijdens levothyroxine suppletie en tijdens onttrekking van schildklierhormoon. Serum en urine osmolaliteit was niet significant verschillend met of zonder levothyroxine behandeling. De studie bevestigt de bekende effecten van schildklierhormoon op serum creatinine gehalte.

Effecten op miroRNAs

In **hoofdstuk 6** werden nieuwe biomarkers voor schildklierhormoonstatus gezocht. Bij 13 patiënten, met in de voorgeschiedenis een gedifferentieerd schildkliercarcinoom, werd de verandering van serum microRNAs onderzocht, na 4 weken onttrekken van levothyroxine en voorafgaand aan radioactief jodiumbehandeling. De huidige studie toont aan dat hypothyreoïdie geen effect heeft op serum microRNAs in mensen en geen geschikte biomarker is voor weefsel specifieke schildkierhormoonstatus.

Klinische factoren van invloed op de behandeluitkomst van schildklierziekten

Medullair schildkliercarcinoom

In **hoofdstuk 7** werd retrospectief het gebruik van peptide receptor radionuclide therapie met ^{177}Lu-octreotaat in 10 patiënten met medullair schildkliercarcinoom met progressieve ziekte of refractaire symptomen onderzocht. Deze studie laat zien dat peptide receptor radionuclide behandeling overwogen kan worden bij patiënten met de combinatie van zowel hoge tumoropname op de ^{111}In-DTPA-octreotide scan (graad ≥3) als positieve somatostatine receptor 2a tumor expressie bij immunohistochemie. Echter, uit ^{111}In-DTPA-octreotide scans verricht bij 35 onbehandelde patiënten met medullair schildkiercarcinoom blijkt dat slechts de minderheid van patiënten voldoende tumoropname heeft om in aanmerking te komen voor peptide receptor radionuclide behandeling.

LIST OF PUBLICATIONS

Beukhof CM, Medici M, van den Beld AW, Hollenbach B, Hoeg A, Visser WE, et al. Selenium Status Is Positively Associated with Bone Mineral Density in Healthy Aging European Men. PLoS One. 2016;11(4):e0152748.

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Beukhof CM, Massolt ET, Visser TJ, Korevaar TIM, Medici M, de Herder WW, et al. Effects of Thyrotropin on Peripheral Thyroid Hormone Metabolism and Serum Lipids. Thyroid. 2018;28(2):168-74.

Reviewed in: H. EC. TSH Has Effects on Peripheral Thyroid Hormone Metabolism That Are Mild but Run Counter to Its Direct Effects on Thyroid Hormone Secretion. Clinical Thyroidology. 2018;30(3):138-41.

Massolt ET, Salih M, **Beukhof CM**, Kam BLR, Burger JW, Visser WE, et al. Effects of Thyroid Hormone on Urinary Concentrating Ability. Eur Thyroid J. 2017;6(5):238-42.

Massolt ET, Chaker L, Visser TJ, Gillis AJM, Dorssers LCJ, **Beukhof CM**, et al. Serum microRNA profiles in athyroid patients on and off levothyroxine therapy. PLoS One. 2018;13(4):e0194259.

Beukhof CM, Brabander T, van Nederveen FH, van Velthuysen MF, de Rijke YB, Franssen GJH, et al. Peptide Receptor Radionuclide Therapy in patients with Medullary Thyroid Carcinoma: predictors and pitfalls (submitted).

Beukhof CM, van Biezen FC, de Herder WW. Slipped capital femoral epiphysis as manifestation of a rare endocrinological disease. Neth J Med;69(2):84, 94.

OTHER PUBLICATIONS

Beukhof CM, Hoorn EJ, Lindemans J, Zietse R. Novel risk factors for hospital-acquired hyponatraemia: a matched case-control study. Clin Endocrinol (Oxf) 2007;66(3):367-72.

Beukhof CM, Lequin MH, Drop SLS. The classic triad and variants. In: Deal C, editor. MRI in Congenital hypopituitarism: a reference guide. London: Remedica; 2007. p.69-80.

PHD PORTFOLIO

Summary of PhD training and teaching

Name PhD student: Beukhof, Carolien M.
Erasmus MC Department: Internal medicine, Endocrinology, Academic Center for Thyroid Diseases

PhD period: 2011-2018 (part-time)
Promotor(s): Prof. Robin P. Peeters, Prof. Wouter W. de Herder

1. PhD training

	Year	Workload (ECTS)
Presentations		
Oral		
- Peptide Receptor Radionuclide Therapy in patients with Medullary Thyroid Carcinoma: predictors and pitfalls. Dutch Endocrine Meeting, Noordwijkerhout, The Netherlands	2018	0.8
- Mechanisms of Sorafenib-induced changes in thyroid hormone levels in patients with hepatocellular carcinoma. Dutch Endocrine Meeting, Noordwijkerhout, The Netherlands	2017	0.8
- Hip complaints as manifestation of an internal disease. European School of Internal Medicine, Brighton, England	2011	0.8
Poster		
- Peptide Peptide Receptor Radionuclide Therapy in patients with Medullary Thyroid Carcinoma: predictors and pitfalls. European Congress of Endocrinology, Barcelona, Spain	2018	0.8
- Effects of thyroid stimulating hormone on serum lipids are mediated via direct effects on peripheral thyroid hormone metabolism. Schidkliersymposium, Rotterdam, The Netherlands	2017	0.8
- Effects of thyroid stimulating hormone on serum lipids are mediated via direct effects on peripheral thyroid hormone metabolism. Dutch Endocrine Meeting, Noordwijkerhout, The Netherlands	2016	0.8

	Year	Workload (ECTS)
- Mechanisms of Sorafenib-induced changes in thyroid hormone levels in patients with hepatocellular carcinoma. European Congress of Endocrinology, Munich, Germany	2016	0.8
- Mechanisms of Sorafenib-induced changes in thyroid hormone levels in patients with hepatocellular carcinoma. Internal Medicine Science days, Antwerp, Belgium	2013	0.8
(Inter)national conferences/courses		
- Teach the teacher	2018	0.3
- European Congress of Endocrinology (2x)	2016, 2018	1.6
- Internistendagen (4x)	2011-2018	3.2
- Dutch Endocrine Meeting (5x)	2011-2018	4.0
- Erasmus Endocrinologie Cursus (3x)	2011-2018	2.5
- Schildklierziekten: een update voor de klinische praktijk	2017	0.2
- Amsterdams Internisten Symposium (2x)	2017, 2018	0.3
- Annual Symposium of the Dutch Thyroid Research F. (2x)	2017, 2018	0.6
- Regionale nascholingsavond Endocrinologie AMC/VUMC (5x)	2016-2018	1.0
- Schildklierkanker anno 2016	2016	0.2
- DOO: hospital management	2014	0.2
- Master Class on Differentiated Thyroid Cancer Management, Pisa, Italy	2013	0.8
- Tumor board meetings, Erasmus MC	2012-2015	1.6
- Attending Endocrinology Lectures, Erasmus MC	2012-2015	0.6
- Continuüm Endocrinologie (2x)	2012, 2013	0.6
- DESG (diabetes) course for fellows in endocrinology	2011	0.3
- Rotterdamse internistendag	2012	0.3
- ESIM summerschool Brighton, England	2011	0.3

2. Teaching

	Year	Workload (Hours/ECTS)
Lectures		
- Lectures on thyroid (dys)function for medical students	2012-2014	0.3
- Lectures on adrenal (dys)function for medical students	2012-2014	0.3
- Chair JNIV opinion session during Dutch national congress of internal medicine	2013	0.3
- Chair landelijke opleidingsdag NIV	2012	0.3
Supervising and tutoring		
- Bed-side teaching interns	2012-2014	0.3
- Tutor medical interns	2012-2014	0.3
- Supervisor internal medicine residency/ advanced nursing practice	2016-2018	3.0
Award		
- Top score Dutch national exam in internal medicine for second year residents	2011	0.1
Other		
- Chair Erasmus MC, young internists	2012-2013	0.8
- National board Dutch society of young internists (JNIV)	2012-2013	0.8
- Dutch representative of young internists on European Federation of Internal Medicine Assembly of Young Internists, Madrid, Spain	2012	0.3

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