TRANSLATIONAL STUDIES TOWARDS UNDERSTANDING CLINICAL EFFECTS OF THYROID HORMONE

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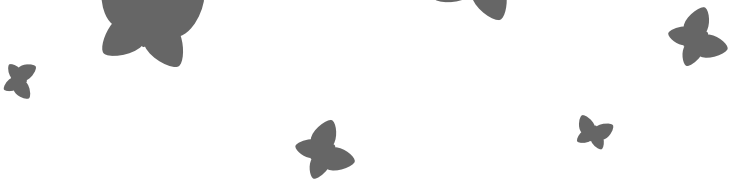

Chapter 1

General introduction

Introduction

Thyroid hormone is indispensable for the metabolism of all tissues. The importance of thyroid hormone in normal physiology is well illustrated by primary thyroid diseases in which abnormal thyroid hormone concentrations affect the function of several organs resulting in a variety of clinical symptoms (1). Hypothyroid symptoms include cold intolerance, constipation, bradycardia and weight gain. In contrast, patients with hyperthyroidism complain of sweating, palpitations and weight loss. These clinical effects of alterations in thyroid state arise from changes in thyroid hormone at the cellular level.

Despite adequate restoration of biochemical euthyroidism with replacement therapy with levothyroxine (LT4), a substantial amount of patients (~10-15%) with hypothyroidism display significant impairment of psychological well-being compared to controls of similar age and sex and show decrements in health status (2-4). In addition, many patients report ongoing symptoms such as muscle aching and tiredness. The reasons for these observations are still unknown. The general focus of this thesis is therefore to explore the cause of persisting symptoms in treated hypothyroid patients and to find new biomarkers for thyroid state.

Thyroid hormone synthesis

Thyroid hormone is synthesised by the thyroid gland, which is located ventrocaudal of the thyroid cartilage. Thyroid hormone synthesis is a multi-step process. First, iodide, the principal component of thyroid hormone is transported across the basolateral membrane of the thyrocyte by the Na/I symporter (NIS). Next, iodide enters the follicular lumen from the cytoplasm by the SLC26A4 transporter (Pendrin). In the colloid, iodide (I^-) is oxidized to iodine ($I^{+)}$ by the enzyme thyroid peroxidase (TPO) and is then condensed onto tyrosine residues which reside along the polypeptide backbone of a protein called thyroglobulin. This reaction results in either a mono-iodinated tyrosine (MIT) or di-iodinated tyrosine (DIT) being incorporated into thyroglobulin. The other synthetic reaction is a coupling reaction, where iodotyrosine molecules are coupled together. If two DIT residues couple together, the result is the formation of 3,3',5,5'-tetraiodothyronine (thyroxine; T4). If a DIT and a MIT residue are coupled together, the result is the formation of 3,3',5-triiodothyronine (T3).

Although T3 is more biologically active than T4, the majority of T3 production (85%) actually occurs outside of the thyroid gland. In peripheral tissues, T3 is produced by conversion from the prohormone T4 by the iodothyronine deiodinases type 1 (D1) and 2 (D2) (Figure 1). D1 is present in liver and kidney, whereas D2 is present in brain, brown adipose tissue, skeletal muscle and the heart. The type 3 deiodinase (D3) is responsible for the degradation of T4 and T3 to the transcriptionally inactive hormones reverse T3 (3,3',5'-triiodothyronine) and 3,3-diiodothyronine (3,3'-T2). D3 is present in brain, skin and placenta.

Although the conversion of T3 to 3,5-diiodothyronine (3,5-T2) has not been experimentally demonstrated in vertebrates, the most plausible pathway for 3,5-T2 formation is the outer ring deiodination of T3 (Figure 1). It is not yet known which deiodinase enzyme catalyses this conversion but a likely candidate is D2 (5).

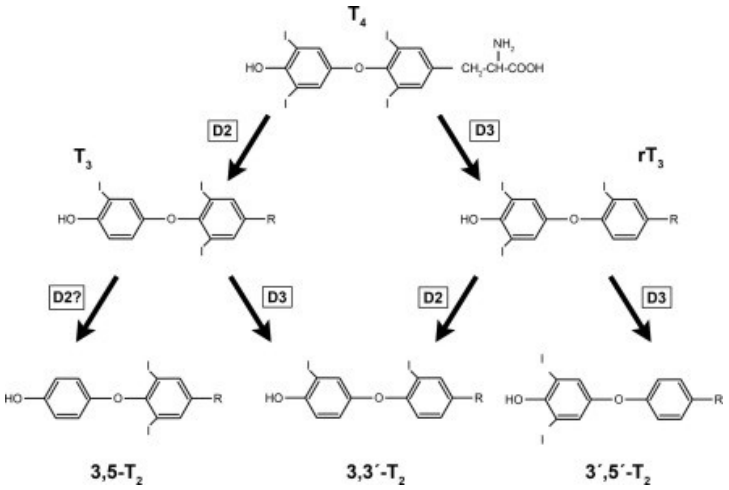

Figure 1. Structures of the principal thyroid hormones and their major pathways of deiodination.

Hypothalamus-pituitary-thyroid axis

Serum thyroid hormone levels are strictly regulated by the hypothalamus-pituitary-thyroid (HPT) axis (6) (Figure 2). Thyroid hormone is secreted in response to thyroid-stimulating hormone (TSH), which is synthesized in the pituitary and mediates its effects via binding to the TSH receptor. TSH production is stimulated by hypothalamic thyrotropin releasing hormone (TRH). The production of TRH and TSH can be downregulated by thyroid hormones (negative feedback). Since serum TSH concentrations reflect the pituitary feedback to serum concentrations of thyroid hormone, it is used in clinical practice to monitor LT4 treatment. However, studies in rodents indicate that different levels of genetic disruption of this feedback mechanism (mice with targeted inactivation of both D1 and D2 genes) have increases in serum T4 and TSH concentrations, while serum T3 concentrations remain stable (7, 8). This implies that the HPT axis is wired to preserve especially serum T3 concentrations (9).

Thyroid hormone action

The cellular uptake of T4 and T3 is mediated by a number of transmembrane transporters. The most important thyroid hormone transporters include the organic anion transporter family member OATP1C1 and monocarboxylate transporter (MCT)8 and MCT10 (10).

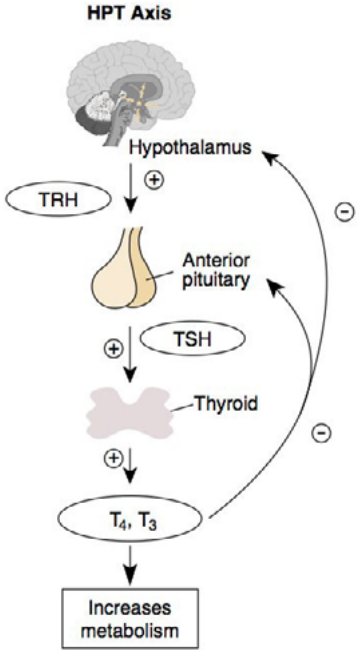

Figure 2. The hypothalamic-pituitary-thyroid axis.

Thyroid hormone acts via genomic and non-genomic pathways. Genomic actions of thyroid hormone are mediated by binding to nuclear T3 receptors (TRs) which regulate transcription of target genes (11). Several receptor isoforms are encoded by the THRA (thyroid hormone receptor α) and THRB (thyroid hormone receptor β) genes of which TRα1, TRβ1 and TRβ2 are the thyroid hormone binding isoforms (12). Both receptors have a wide expression pattern, with a predominance of TRα1 in brain, heart and bone and TRβ1 as the major isoform in the liver, kidney and thyroid. TRβ2 is mainly expressed in hypothalamus and pituitary and is therefore involved in the regulation of the HPT axis (13). The TRs functions as ligand-dependent transcription factors. TRs bind to thyroid hormone response elements (TREs) in the promoter regions of target genes, thereby regulating gene transcription by the interaction with co-activators and co-repressors (12). Many different animal models have been used to explore which genes are regulated by thyroid hormone (14-16).

However, it is largely unknown which genes are regulated by thyroid hormone in human tissues. More knowledge of the effects of thyroid hormone on gene expression in different tissues would lead to a better understanding of the widespread clinical effects of thyroid hormone in the human body. In a previous study of 10 thyroidectomised patients with differentiated thyroid carcinoma (DTC) 607 differentially expressed genes in skeletal muscle samples on and after 4 weeks off LT4 replacement were detected, of which approximately 60%

were positively and approximately 40% were negatively regulated (17). This study also showed that the expression of the precursor microRNA (miRNA) pair miRNA-206/ miRNA-133b highly differed on thyroid state. MiRNAs are small non-coding RNA molecules, which post-transcriptionally repress the expression of target genes by binding to the 3'UTR of messenger RNA (Figure 3) (18-20). MiRNAs exhibit tissue-specific expression patterns and each miRNA may affect the expression of hundreds of target genes. Remarkably, miRNAs are present in the circulation and have been associated with a variety of diseases (21, 22).

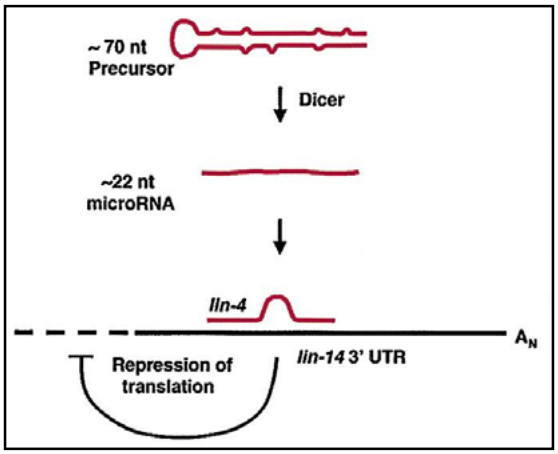

Figure 3. A canonical microRNA, lin-4, is processed from its precursor transcript by Dicer, and base pairs with a complementary sequence in the 3′UTR of a target, in this case lin-14 mRNA, thereby triggering a cis-acting translational repressive activity. (image adapted from Ambros *et al* (20))

Hypothyroidism

Thyroid autoimmunity is the most common cause of hypothyroidism (Hashimoto's disease). TPO antibodies (TPO-Abs) are present in almost all patients with autoimmune hypothyroidism (1). Autoimmune hypothyroidism is commonly accompanied by depressive symptoms. A large epidemiological Danish nationwide, prospective cohort study showed that various autoimmune diseases including autoimmune thyroid disease (AITD), are associated with subsequent lifetime mood disorder diagnosis (e.g. bipolar affective disorder, unipolar depression, psychotic depression and other remaining mood disorders) (23). Even subjects with thyroid autoimmunity but still with a normal thyroid function have a higher risk to develop mood disorders (24). Also offspring of patients with a bipolar disorder have a higher prevalence of TPO-Abs, even if they are not affected by the disorder (25). In addition, a higher prevalence of AITD has been reported in patients with bipolar disorders, irrespective of the usage of lithium (26). Taken together, these associations might imply a shared immune pathogenesis for both AITD and mood disorders which needs to be explored.

However, after thyroidectomy, cured patients with differentiated thyroid carcinoma (DTC) on LT4 replacement therapy have also been shown to have an impaired quality of life (QoL) although they are not affected by an autoimmune disease (27, 28). An explanation for this impaired QoL might be the inadequacy of LT4 treatment to restore physiological T4 and T3 concentrations in serum and tissues (29, 30). In euthyroid patients, approximately 15% of circulating T3 is directly secreted by their thyroid gland. Nevertheless, in hypothyroid patients, LT4 monotherapy is the standard of care. The peripheral conversion of LT4 to T3 makes it possible to achieve normal T3 concentrations in patients treated with LT4, albeit with the necessity for maintaining T4 concentrations at the higher end of the normal range (31-33). Studies in hypothyroid rats have demonstrated that LT4 monotherapy is not able to normalize T4 and T3 concentrations in all tissues (34). 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. However, addition of synthetic T3 (liothyronine) to LT4 treatment regimens is controversial. A meta-analysis of 11 studies that had been performed so far comparing the effectiveness of T4-T3 combination therapy vs LT4 monotherapy, did not find any improvement of symptoms and QoL for T4-T3 combination therapy (35). It should be mentioned that the wide variation in T4 to T3 dose ratio within and between these trials constitutes a potential bias because most studies fail to mimic the ratio of T4 to T3 secretion by the thyroid gland under physiological conditions (36). Furthermore, synthetic T3 was used in the plain form in all these studies (a reliable slow-release T3 is currently not (yet) available), resulting in non-physiologic serum T3 peaks (37). Substitution with T3 should preferably be performed with a preparation that slowly releases T3 to avoid these peaks (38). Also, it remains unknown whether other endogenous thyroid hormone metabolites with T3-agonistic or antagonistic activities, like 3,5-T2, might play a role in the persisting symptoms and decreased QoL among hypothyroid patients. Recent studies have shown that 3,5-T2 concentrations are higher in thyroidectomized patients on LT4 substitution therapy as compared with healthy controls, suggesting extrathyroidal production or conversion (39).

Finally, several studies have shown that serum TSH, which is currently used to monitor LT4 treatment, is not always an appropriate marker and that normal serum TSH concentrations in patients receiving LT4 reflect only pituitary euthyroidism (40-42). For all these reasons, novel markers representing tissue thyroid state are needed.

Outline of the thesis

The studies documented in this thesis consist of three major parts. The first part of the thesis (**chapters 2 and 3**) includes 2 studies on the physiological effects of thyroid state on target tissues/organs. In **chapter 2**, the effect of thyroid state on renal concentrating ability was studied in DTC patients on and off LT4 treatment. We used serum and urine samples of DTC patients because they are subject to severe hypothyroidism before radioactive iodine therapy to stimulate radioactive iodine uptake by malignant tissues, whereas they have relatively high thyroid hormone concentrations afterwards when receiving substitution therapy with LT4 in order to reach TSH suppression. In **chapter 3**, the effect of thyroid state on the microcirculation was studied in other DTC patients on and off LT4 treatment. The microcirculation is the main site of oxygen delivery to tissue cells and is essential for the maintenance of cellular life and function. The function of the organs is directly dependent on the function of their respective microcirculation. The effects of thyroid hormone deficiency on the microcirculation have not been well assessed.

In the second part of the thesis, we explored the link between AITD and mood disorders (**chapter 4**) and studied the association between thyroid hormones and QoL (**chapter 5**). To explore the link between AITD and mood disorders we determined 5 growth and differentiation factors that have repeatedly been shown to be abnormally expressed in the circulation of mood disorder patients and that are capable of influencing immune and/or neuronal cell growth (**chapter 4**). In **chapter 5**, we studied the association between extensive thyroid function tests, including 3,5-T2 concentrations and T4/T3 ratios, and QoL in athyreotic patients on LT4 replacement. We studied patients with DTC, as these patients lack endogenous thyroidal T3 secretion after thyroidectomy in the absence of an autoimmune disease.

In the third part of this thesis (**chapters 6 and 7**), we searched for new biomarkers of thyroid state. **Chapter 6** describes an attempt to measure potential miRNAs in serum regulated by thyroid state. To study the effects of thyroid state on gene expression in whole blood (mainly consisting of TRα expressing leucocytes) we performed next generation RNA sequencing in DTC patients on and off LT4 treatment (**chapter 7**).

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PART I

Physiological effects of thyroid hormone

Chapter 2

Effects of thyroid hormone on renal concentrating ability

Elske T. Massolt, Mahdi Salih, Carolien M. Beukhof, Boen L.R. Kam, J.W. Burger, W. Edward Visser, Ewout J. Hoorn, Robin P. Peeters

Eur Thyroid J. 2017; 6:238–242

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, Den Haag, 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 25^{th} and 75^{th} 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 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).

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 (25^{th}-75^{th} 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).

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

Data are expressed as median (25^{th}-75^{th} percentile).

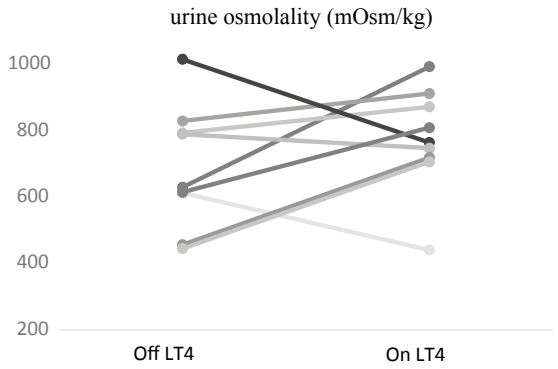

Figure 1. Changes in urine osmolality off and on LT4 treatment. Each line between two dots represents a patient.

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 3

The effects of thyroid state on microcirculation and macrocirculation

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Submitted

Abstract

Objective*:* Normal thyroid hormone (TH) levels are required to maintain cardiovascular function. Changes in thyroid state affect global hemodynamic parameters, but it is less clear if and how it affects the microcirculation. The direct relationship between TH effects on macrocirculation and microcirculation has never been studied. We report a full integrated assessment of macrocirculation and microcirculation in patients with different thyroid states.

Design and methods: We studied 10 thyroidectomized patients (4 males) on and after 4 weeks off levothyroxine replacement. Macrocirculation was assessed by measuring standard hemodynamic parameters and transthoracic echocardiography. Peripheral perfusion was documented by measuring the body temperature gradient and performing transcutaneous oximetry and near-infrared spectroscopy. Using an Incident Dark Field Imaging based handheld microscope the sublingual (central) microcirculation was quantified.

Results: Heart rate, pulse pressure, end diastolic volume and stroke volume significantly decreased, while median systemic vascular resistance increased during hypothyroidism. Forearm to-fingertip skin-temperature gradient was significantly higher and the peripheral perfusion index was significantly lower during hypothyroidism. Parameters of the sublingual microcirculation did not differ between thyroid states.

Conclusion: Our study confirms several well-known effects of TH on cardiovascular function. In addition, we observed that hypothyroidism had profound effects on peripheral perfusion, but not on central microcirculation, which may be explained as an adaptive mechanism for the reduced thermogenesis. Together, our studies reinforce that TH exerts its well-known clinical effects on cardiovascular systems through different physiologic mechanisms.

Introduction

Thyroid hormone (TH) affects virtually all tissues and cells in the body. Prominent features of thyrotoxicosis include palpitations and heat intolerance, while hypothyroidism is accompanied by bradycardia and cold intolerance. These clinically well-recognized features of abnormal TH levels highlight the important effects of TH on normal cardiovascular function. Triiodothyronine (T3), the physiologically active form of TH, controls the inotropic properties of the myocardium, cardiac growth and vascular function via genomic and non-genomic actions (1-4).

TH effects on the heart and peripheral vasculature include decreased systemic vascular resistance (SVR) and increased resting heart rate (HR), left ventricular contractility, and blood volume (5). As a corollary, hypothyroidism results in bradycardia, decreased ventricular filling and cardiac contractility, which together decreases cardiac output.

Although effects of TH on the macrocirculation have been studied extensively, much less is known on the effects of TH on the microcirculation in humans (4-6). The microcirculation is the last step of oxygen delivery to tissues and cells and is, therefore, essential for the maintenance of cellular life and function. The microcirculation can be defined as vessels smaller than 100 microns, i.e., arterioles, capillaries and venules and facilitates fine-tuning of perfusion to meet local oxygen requirements (7). Microcirculatory derangements with severe consequences have been shown in sepsis, hypovolemia and cardiac failure (8, 9). Effects of TH on the microcirculation have been demonstrated in a hamster cheek pouch model (10). The addition of NG-nitro-L-arginine methyl-ester (L-NAME), which is an inhibitor of nitric oxide (NO) synthase, counteracted the T3-induced dilation, suggesting that the effects of T3 on the *in vivo* microcirculation appear to be mediated by NO through a non-genomic mechanism of action (10).

In humans, peripheral microcirculation can be assessed by measuring the body temperature gradient and performing transcutaneous oximetry and near-infrared spectroscopy (NIRS), while central microcirculation can be studied with handheld video-microscopes (11, 12). Previous studies have documented the impact of thyroid state on various parameters of macrocirculation and microcirculation (13, 14). However, none of the studies have simultaneously assessed macrocirculation and microcirculation. Therefore, a direct comparison of the effects of TH on macrocirculation and microcirculation is currently lacking. The aim of this study was to perform a full integrated assessment of macrocirculation and microcirculation with well-established and state-of-the-art tools in athyreotic patients off and on levothyroxine (LT4) replacement therapy.

Subjects and methods

Patients with differentiated thyroid cancer (DTC), 18-70 years old, were recruited from the outpatient clinic of the Erasmus Medical Center Rotterdam, between June 2015 and March 2016. Initial therapy consisted of total thyroidectomy. Patients were eligible for inclusion if they were scheduled for ablation with radioactive iodine (RAI), did not have heart failure or drugs interfering with TH metabolism. The first measurement was scheduled after four weeks off LT4 (i.e. LT4 withdrawal before RAI ablation) and the second measurement after about 3 months on LT4 replacement therapy once TSH-suppression was reached. The Medical Ethics Committee of the Erasmus Medical Center approved the study protocol (MEC-2014-489) and written informed consent was obtained from all study participants.

Laboratory measurements

Serum free T4 (FT4), total T4 and total T3 concentrations were measured by chemo luminescence assays (Vitros ECI Immunodiagnostic System; Ortho-Clinical Diagnostics, Rochester, MI). Serum TSH was measured by immunometric assay (Immulite 2000 XPi, Siemens, Den Haag, the Netherlands). Creatine kinase (CK), CK-MB, lactate, and aspartate aminotransferase (AST) were measured by Roche/Hitachi cobas c501. N-terminal-pro-B-type natriuretic peptide (NT-proBNP) was measured by an electrochemiluminescence immunoassay (Cobas e 601/602).

Macrocirculatory function

Transthoracic echocardiography using an iE33 Ultrasound System (Philips Healthcare, Amsterdam, the Netherlands) was performed by experienced technicians who were blinded to the thyroid state of the subjects. We assessed left ventricular end diastolic volume (EDV) and end systolic volume (ESV) and left atrial volume with biplane Simpson's method (TomTec Cardiac Performance Analysis ©, Munich, Germany). Stroke volume (SV = EDV - ESV), cardiac output (CO = HR x SV/1000) and ejection fraction (EF = 100 x (EDV - ESV/EDV)) were subsequently calculated. Mitral inflow velocity was derived from the pulsed-wave Doppler in the apical four-chamber view: early transmitral inflow (E) velocity, its decelerating time (DET) and late transmitral flow (A) velocity were assessed and then the E/A ratio was calculated. By using pulsed-wave tissue Doppler image (TDI), early diastolic (E′) mitral annular velocity was obtained at the septal mitral annulus level in the apical four-chamber view with septal annulus movement aligned with the sample volume line, and the E/E′ ratio was derived.
We assessed systolic blood pressure (SBP) and diastolic blood pressure (DBP) and calculated mean arterial blood pressure (MAP) and pulse pressure (PP = SBP - DBP). The SVR was calculated by the formula: 80 x (MAP - right atrial pressure)/CO. Cardiac conduction times were measured with a 12-lead electrocardiogram (ECG).

Peripheral microcirculatory function

Capillary refill time (CRT) was assessed, which is defined as the time required for the nail bed to regain its color after pressure has been applied to cause blanching.

Forearm to-fingertip skin-temperature gradient (Tskin-diff) has also been used as an index of peripheral circulation (15). Tskin-diff is obtained with two skin probes (Hewlett Packard 21078A) attached to the index finger and on the radial side of the forearm, midway between the elbow and the wrist. When vasoconstriction decreases finger-tip blood flow, finger skin temperature decreases, and Tskin-diff increases (11). A Tskin-diff threshold of 0 °C for initiating vasoconstriction and of 4 °C for severe vasoconstriction have been suggested (16).

The peripheral perfusion index (PFI) was derived from the photoelectric plethysmographic signal of the finger pulse oximeter. This noninvasive tool uses two wavelengths of light (red and infrared) that are transmitted through the distal phalanx of the index finger, resulting in the display of a pulsatile photoplethysmographic waveform. The PFI is calculated as the ratio between the pulsatile component (arterial compartment) and the non-pulsatile component (others tissues) of the light reaching the detector of the pulse oximeter. A PFI of 1.4 or lower has been found to be correlated best with hypoperfusion in critically ill patients (11).

NIRS uses the principles of light transmission and absorption to measure the concentrations of hemoglobin and oxygen saturation (StO2) noninvasively in tissues (11). NIRS provides a global assessment of oxygenation in all vascular compartments (arterial, venous, and capillary). An inSpectra tissue spectrometer model 650 with a 15-mm probe was placed over the thenar eminence to measure StO2.

Assessment of the sublingual/central microcirculation

We used Incident Dark Field (IDF) technology (CytoCam, Braedius Medical, Amsterdam, NL) to visualise the sublingual microcirculation (17). In brief, it consists of a handheld video microscope that emits stroboscopic green light (530 nm) from an outer ring at the end of a probe, which is absorbed by haemoglobin. Thus, a negative image of moving red blood cells is transmitted back through the isolated optical core of the probe toward a charge coupled device camera. We adhered to the recommendations from an expert panel on how to evaluate the microcirculation using SDF imaging (18).

At each time point, clips at 3 different sublingual sites yielding at least 20 sec of stable video per site were recorded. All clips were stored under a random number, which were analysed by one trained and blinded observer using dedicated software (AVA 3.0, Microvision Medical, NL). Microvascular flow index (MFI), total vessel density (TVD), perfused vessel density (PVD) and proportion of perfused vessels (PPVs) were determined for every patient at the two different time points. All scores have been validated previously (19). For PPV and PVD, vessel density was calculated as the number of vessels crossing 3 horizontal and 3 vertical equidistant lines, spanning the screen divided by the total length of the lines. Perfusion at each crossing

was then scored semi-quantitatively as follows: 0 = no flow (no flow present for the entire duration of the clip), 1 = intermittent flow (flow present <50% of the duration of the clip), 2 = sluggish flow (flow present >50% but <100% of the duration of the clip or very slow flow for the entire duration of the clip), and 3 = continuous flow (flow present for the entire duration of the clip). PVD was calculated as the number of crossings with flow scores greater than 1. PPV was calculated as the proportion of crossings with flow scores greater than 1 divided by the total number of crossings. Each score was determined for small microvessels with a cut-off diameter of 25 μm. In previous studies, intra-observer variability ranges between 2.5 and 9.3% for PVD and between 0 and 4.5% for PPV and inter-observer variability ranges between 3.0 and 10.1% for PVD and between 0 and 10% for PPV (19). MFI was based on the determination of the predominant type of flow in 4 quadrants. MFI is the sum of these flow scores divided by the number of quadrants in which the vessel type is visible. For each time point and each patient, all scores were averaged.

Statistical analysis

Data were expressed as median with inter quartile range (IQR). 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). A p value < 0.05 was considered as statistically significant. Since most parameters were interrelated we did not correct for multiple testing.

Results

The baseline characteristics of the 10 included patients are shown in Table 1. As expected, all thyroid function tests were significantly different off and on LT4 treatment (Table 2), reaching deeply hypothyroid levels in patients off LT4. CK and ASAT levels were significantly higher, while NT-proBNP levels were significantly lower off LT4 treatment.

Table 1. Characteristics of study participants

Sex *N(%)*	
Male	4 (40.0)
Female	6 (60.0)
Age (years)	43.0 (30.2-55.8)
Time between tests (weeks)	12.2 (11.0-15.9)
Dose LT4 (μg)	187.5 (150.0-225.0)
Dose LT4 (μg/kg)	2.1 (1.8-2.6)
BMI (kg/m²)	28.0 (23.0-31.8)

Table 2. Changes in thyroid function tests and in several serum biomarkers of organ function off and on LT4 treatment

	Reference range	Off LT4		On LT4		*P* value
TSH	0.4-4.3 mU/L	99.8	(67.0-144.0)	0.07	(0.01-0.24)	0.005
FT4	11.0-25.0 pmol/L	1.3	(0.9-1.9)	32.0	(22.8-35.9)	0.005
Total T4	58.0-128.0 nmol/L	13.0	(6.5-18.5)	163.0	(134.0-185.5)	0.008
Total T3	1.4-2.5 nmol/L	0.63	(0.42-0.9)	1.98	(1.8-2.2)	0.005
Lactate	0.5-1.7 mmol/L	1.70	(1.4-1.8)	1.45	(1.0-1.6)	0.085
CK	Males < 171, females < 145 U/L	185.0	(83.8-493.5)	106.0	(52.5-196.0)	0.005
CK-MB	Males < 7.6, females < 4.7 µg/L	3.5	(2.1-5.9)	2.2	(1.4-3.5)	0.075
ASAT	Males < 35, females < 31 U/L	43.0	(24.0-52.5)	23.0	(17.8-35.5)	0.025
NT-ProBNP	< 15.0 pmol/L	1.0	(1.0-1.5)	6.0	(2.5-8.5)	0.012

Data are expressed as median (IQR).

Global hemodynamic parameters are shown in Table 3. Heart rate, systolic blood pressure and pulse pressure were significantly lower during hypothyroidism. Echocardiography revealed that EDV, left atrial volume, CO and SV were significantly smaller and that SVR was significantly higher in patients off LT4 (Figure 1). Hypothyroidism caused a significant reduction of peak velocity at early mitral inflow (E-top), while E/A ratio, E/E' and DET were not significantly different off and on LT4 treatment (Table 4).

Table 3. Changes in global hemodynamic parameters and QTc off and on LT4 treatment

	Off LT4		On LT4		*P* value
Heart rate (beats/min)	53.5	(46.8-64.8)	65.5	(58.3-69.8)	**0.017**
Systolic blood pressure (mmHg)	128.5	(116.0-140.0)	133.0	(127.0-141.8)	**0.032**
Diastolic blood pressure (mmHg)	85.5	(74.5-91.5)	79.0	(73.0-82.8)	0.24
MAP (mmHg)	100.0	(87.7-108.4)	95.8	(91.0-102.9)	0.80
Pulse pressure (mmHg)	46.5	(38.3-50.3)	55.0	(53.0-60.5)	**0.005**
Cardiac output (l/min)	3.6	(3.2-4.2)	5.1	(4.0-5.8)	**0.005**
SVR (dynes-sec/cm-5)	1972	(1730-2409)	1626	(1302-1827)	**0.007**
Ejection fraction (%)	57	(50-58)	56	(51-61)	0.65
SpO2 (%)	97.5	(96.0-100.0)	97.5	(96.8-99.0)	0.34
QTc (msec)	390.5	(372.0-404.3)	385.5	(372.3-412.8)	0.29

Data are expressed as median (IQR). MAP, mean arterial pressure; SVR, systemic vascular resistance; SpO2, peripheral oxygen saturation.

Table 5 shows the central microcirculation and peripheral circulation parameters. The Tskin-diff was significantly higher during hypothyroidism, which was driven by lower temperatures measured on the finger compared with the forearm. The PFI was significantly lower during hypothyroidism, but StO2 was unchanged. Video-assisted assessment of the sublingual microcirculation did not reveal differences between patients off and on LT4 treatment.
To assess if the changes in peripheral perfusion resulted from macrocirculatory changes, we sought to correlate the individual changes in microcirculatory and macrocirculatory parameters. No correlation was observed, suggesting that TH-dependent microcirculatory changes are independent of the macrocirculation (data not shown).

Table 4. Doppler-echocardiographic parameters off and on LT4 treatment.

	Off LT4		**On LT4**		***P* value**
E-top (cm/sec)	0.58	(0.42-0.68)	0.71	(0.51-0.82)	0.028
E/A ratio	1.38	(0.6-2.0)	1.31	(0.8-1.8)	0.22
E/E' ratio	8.4	(6.9-9.6)	8.9	(7.9-9.9)	0.48
DET (msec)	195.0	(175.0- 265.0)	195.5	(168.8-264.5)	0.54

E-top, early transmitral inflow velocity; A, late transmitral flow velocity; E′, early diastolic mitral annular velocity; DET, decelerating time. Data are expressed as median (IQR).

Table 5. Parameters of the sublingual (central) microcirculation and of peripheral circulation off and on LT4 treatment.

	Off LT4		**On LT4**		***P* value**
Sublingual microcirculation					
TVD (mm/mm2)	17.2	(15.7-19.6)	16.9	(14.4-20.4)	0.8
PVD (mm/mm2)	16.5	(13.7-18.3)	15.0	(13.7-18.6)	0.96
PPV (%)	91.3	(86.3-96.8)	91.9	(82.3-97.0)	0.96
MFI	2.67	(2.4-3.0)	2.75	(2.6-2.9)	0.59
Peripheral circulation					
StO2 (%)	74.5	(70.0-83.3)	78.0	(73.5-81.3)	0.48
PFI (%)	1.7	(0.95-3.7)	2.9	(1.7-5.1)	0.037
Tskin-diff (°C)	2.75	(-0.02-5.3)	1.1	(-1.0-3.6)	0.047
CRT (sec)*	4.0	(3.0-4.8)	3.0	(3.0-4.6)	1.0

Data are expressed as median (IQR). TVD, total vessel density; PVD, perfused vessel density; PPV, proportion of perfused vessels; MFI, microvascular flow index; StO2, tissue oxygen saturation; PFI, peripheral perfusion index; Tskin-diff, Forearm to-fingertip skin-temperature gradient; * Capillary refill time (CRT) was assessed in 9 patients because one patient had artificial nails.

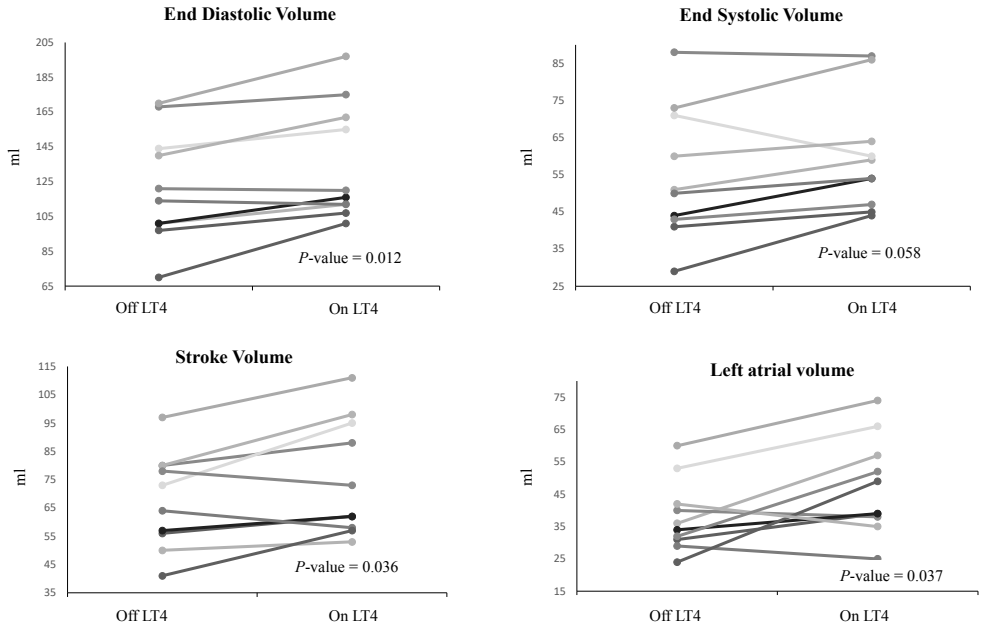

Figure 1. Changes in several echocardiographic parameters off and on LT4 therapy. Each line between two dots represents a patient. P-values represent non-parametric Wilcoxon signed rank test.

Discussion

Although the effects of TH on the cardiovascular system are well-known, the present study documented for the first time simultaneously macrocirculation and microcirculation in humans in different thyroid states.

Several well-known consequences of hypothyroidism on the macrocirculation were confirmed, including a narrowing of pulse-pressure, decreased heart rate and lower left EDV, left atrial volume, CO and SV (5, 6). Together, these changes explain the increase in SVR, concordant with early studies (2). The lower early trans mitral inflow (E-top) velocity during hypothyroidism is consistent with a reduction of pre-load. Although clinical and subclinical hypothyroidism are associated with diastolic dysfunction that can improve upon LT4 replacement therapy, we did not observe changes in Doppler-derived diastolic function parameters (5). This might be explained by the fact that in our study the duration of the hypothyroid state was relatively short compared to other studies (20-22). Indeed, other studies on short-term hypothyroidism did not report diastolic dysfunction either (23, 24).

The increase in Tskin-diff and the decrease in PFI represent strong effects on the very peripheral circulation upon different thyroid state. Similar findings have been reported previously using different techniques (13, 14, 25, 26). Theoretically, there are a few scenarios that potentially explain the decreased peripheral circulation in hypothyroidism. First, CO could be insufficient

to maintain adequate peripheral perfusion. However, this is less likely as the decrease in CO is not as low as typically seen in conditions where decreased CO does affect the peripheral circulation (e.g. cardiac forward failure) (11, 27, 28). Furthermore, we did not observe correlations between changes in global hemodynamic parameters and changes in Tskin-diff or PFI. Second, amelioration of direct effects of TH on the vessel walls in hypothyroidism might be an explanation. However, StO2 representing small arteries (hand) and sublingual microcirculation are both unaffected. Therefore, a general effect on small blood vessels is less likely. Third, the decreased peripheral circulation may be secondary to TH-dependent changes in thermoregulation. Given the effects of TH on thermoregulation, cutaneous vasoconstriction can be a partial compensation for the reduced central thermogenesis and, thus, may have accounted for the decrease in peripheral perfusion in our patients (13, 25, 29). The latter possibility would also explain the absence of changes in the sublingual (central) microcirculation as this is not involved in (peripheral) thermoregulation.

Our study has certain limitations that need to be taken into account when considering the results. First, the number of included patients is small, which is a consequence of the steadily growing number of DTC patients that are treated with radioactive iodine after preparation with recombinant TSH instead of LT4 withdrawal (30). However, considering the fact that all patients were deeply hypothyroid, a clinically relevant effect on the sublingual microcirculation should have been detected. Second, although patients reached a deeply hypothyroid state, the duration of true hypothyroidism was relatively short. This limits the detection of changes that occur during longstanding (subclinical) hypothyroidism. Finally, since all our subjects were athyreotic, which can lead to changes in the physiological T3/T4 ratio if levothyroxine is supplemented, our results do not necessarily reflect the effect of endogenously produced thyroid hormones (31). In addition, several strengths should be mentioned. First, we performed a fully integrated assessment of microcirculation and macrocirculation with well-established and state-of-the-art tools. Second, the study design included paired analyses, which has the advantage to reduce confounders.

In conclusion, we confirmed several well-known effects of TH on cardiovascular function. In addition, we observed that hypothyroidism had profound effects on peripheral perfusion, but not on central microcirculation, which is potentially explained as an adaptive mechanism for the reduced thermogenesis. Together, our studies reinforce that TH exerts its well-known clinical effects on cardiovascular systems through different physiologic mechanisms.

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PART II

Cognitive effects of thyroid hormone

Chapter 4

Aberrant levels of hematopoietic/ neuronal growth and differentiation factors in euthyroid women at risk for autoimmune thyroid disease

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Abstract

Background: Subjects at risk for major mood disorders have a higher risk to develop autoimmune thyroid disease (AITD) and vice-versa, implying a shared pathogenesis. In mood disorder patients, an abnormal profile of hematopoietic/neuronal growth factors is observed, suggesting that growth/differentiation abnormalities of these cell lineages may predispose to mood disorders. The first objective of our study was to investigate whether an aberrant profile of these hematopoietic/neuronal growth factors is also detectable in subjects at risk for AITD. A second objective was to study the inter relationship of these factors with previously determined and published growth factors/cytokines in the same subjects.

Methods: We studied 64 TPO-Ab-negative females with at least 1 first- or second-degree relative with AITD, 32 of whom did and 32 who did not seroconvert to TPO-Ab positivity in 5-year follow-up. Subjects were compared with 32 healthy controls (HCs). We measured serum levels of brain-derived neurotrophic factor (BDNF), Stem Cell Factor (SCF), Insulin-like Growth Factor-Binding Protein 2 (IGFBP-2), Epidermal Growth Factor (EGF) and IL-7 at baseline.

Results: BDNF was significantly lower (8.2 vs 18.9 ng/ml, $P<0.001$), while EGF (506.9 vs 307.6 pg/ml, $P=0.003$) and IGFBP-2 (388.3 vs 188.5 ng/ml, $P=0.028$) were significantly higher in relatives than in HCs. Relatives who seroconverted in the next 5 years had significantly higher levels of SCF than non-seroconverters (26.5 vs 16.7 pg/ml, $P=0.017$). In a cluster analysis with the previously published growth factors/cytokines SCF clustered together with IL-1β, IL-6 and CCL-3, of which high levels also preceded seroconversion.

Conclusion: Relatives of AITD patients show aberrant serum levels of 4 hematopoietic/neuronal growth factors similar to the aberrancies found in mood disorder patients, suggesting that shared growth and differentiation defects in both the hematopoietic and neuronal system may underlie thyroid autoimmunity and mood disorders. A distinct pattern of four inter correlating immune factors in the relatives preceded TPO-Ab seroconversion in the next 5 years.

Introduction

Autoimmune hypothyroidism is characterized by a combination of clinical features, elevated serum TSH with reduced free T4 (FT4) levels, the presence of serum antibodies against thyroid antigens, and reduced echogenicity of the thyroid sonogram (1). It is the most common organ-specific autoimmune disorder with an estimated prevalence of 2%, with a higher prevalence in women and depending on iodine intake (2-5). Thyroid peroxidase (TPO) is the major autoantigen and TPO antibodies (TPO-Abs) are present in almost all patients with autoimmune hypothyroidism (6) and precede the clinical phase of autoimmune hypothyroidism by many years. Subclinical autoimmune hypothyroidism (the presence of TPO-Abs with raised TSH and normal FT4 levels) is even more prevalent and affects about 9% of the population (2, 5). In the Whickham follow-up study, women with TPO-Abs had an eight-fold higher risk of developing clinically overt hypothyroidism over 20 years than did antibody-negative women (7). In our own studies on the Amsterdam AITD cohort (euthyroid females with at least one first or second degree relative with a documented autoimmune hyper- or hypothyroidism) TPO-Ab positivity at the start of the study also represented a higher risk to develop overt hypothyroidism in a follow-up of 5 years (8, 9). In addition, there was a higher conversion rate from TPO-Abs negativity to positivity, showing a familial proneness for thyroid autoimmune reactivity (9, 10).

In another previous study on this cohort, we tested the hypothesis that serum levels of factors related to thyroid growth and connective tissue abnormalities (Platelet-Derived Growth Factor (PDGF)-BB, Fibronectin, Metalloproteinase (MMP)-13), to the early accumulation of immune cells in the thyroid (soluble Vascular Cell Adhesion Molecule (sVCAM)-1, CCL2, CCL4, Angiopoetin-1 Receptor-2 (TIE-2)), and to inflammation (IL-1β, IL-6 and CCL3) were related to this proneness for thyroid autoimmunity in the relatives (11). We therefore studied these factors in the serum of 64 TPO-Ab negative euthyroid relatives, 32 of whom did and 32 of whom did not seroconvert to TPO-Abs positivity in 5 year follow-up. The relatives were compared with 32 healthy controls. We found that both seroconverting and non-seroconverting relatives showed an up regulation of Fibronectin and a down regulation of PDGF-BB, CCL2, CCL4, sVCAM-1, TIE-2 and MMP-13. The relatives who later seroconverted (seroconverters, SCs) differed from those who did not seroconvert (non-seroconverters, NSCs) by a significant up regulation of pro-inflammatory compounds, such as IL-1β, IL-6 and CCL3. We concluded that euthyroid females within AITD families show a characteristic pattern of abnormalities in serum levels of growth factors, chemokines, adhesion molecules and cytokines, suggesting an already compromised thyroid-immune system interaction in the euthyroid family members. Also, pre-seroconversion stages might be predicted using serum analytes pointing to a higher inflammatory state.

Autoimmune hypothyroidism is commonly accompanied by depressive symptoms. A large epidemiological Danish nationwide, prospective cohort study showed that various autoimmune diseases including AITD, are associated with subsequent lifetime mood disorder diagnosis (e.g. bipolar affective disorder, unipolar depression, psychotic depression and other remaining mood disorders) (12). In hypothyroid patients the lack of thyroid hormone in the brain is likely an important determinant for these mood disturbances (13). However, a deficit of thyroid hormone may not be the only cause, as even subjects with TPO-Abs with normal thyroid function have a higher risk to develop anxiety disorders and mood disorders (14). Also offspring of patients with a bipolar affective disorder have a higher prevalence of TPO-Abs, even if they are not affected by the psychiatric disorder (15, 16). In addition, a higher prevalence of TPO Abs and autoimmune hypothyroidism has been reported in patients with bipolar affective disorder, irrespective of the usage of lithium (17, 18). Taken together, these associations might imply a shared immune pathogenesis for both AITD and mood disorders. We therefore additionally determined, in the sera used in the previous study, 5 growth and differentiation factors that have *repeatedly* been shown to be abnormally expressed in the circulation of mood disorder patients and that are capable of influencing both immune and/or neuronal cell growth, i.e. SCF, IGFBP-2, EGF, BDNF and IL-7 (19-23). In addition we studied the inter relationship of these factors with the previously determined factors using a cluster analysis to study patterns of TPO-Ab seroconversion (11).

Subjects and methods

Subjects

The Amsterdam AITD cohort has previously been described in detail (8). In the present study, we studied serum levels of several hematopoietic/neuronal growth factors in the subjects. In addition we assessed the association with TPO-Ab seroconversion like we previously did in the study of Beumer *et al* (11). Therefore, 32 euthyroid subjects were selected who were TPO-Ab and Tg-Ab negative at baseline but developed TPO-Abs during follow-up without developing an abnormal TSH. Each selected SC was matched with an euthyroid subject who was TPO-Ab and Tg-Ab negative at baseline and did not develop TPO-Abs (non-seroconverter) up to the time at which the SC to whom they were matched had received her endpoint. SCs and NSCs were matched for age, current smoking, current estrogen use, and duration of follow-up.
As a control group, we selected 32 female subjects from an ongoing program for delineating reference values of endocrine function tests that were in self-proclaimed good health, were not using chronic medication (except for oral contraceptives), had no family or personal history of thyroid disease, and had normal TSH and no thyroid antibodies. Blood samples were collected over the same period of time as those of the Amsterdam AITD cohort and were processed in the same manner.

All subjects gave informed written consent and the Medical Ethics Committee of the Academic Medical Center in Amsterdam and the Medical Ethics Committee of Erasmus Medical Center in Rotterdam approved the study.

TSH, FT4 and TPO-Ab determinations

Serum samples were stored at −20°C until determination of the study parameters. Serum TSH and FT4 were measured using time-resolved fluoroimmunoassay (Delphia, Turku, Finland). Reference values are 0.4–5.7 mU/L for TSH and 9.3–20.1 pmol/L for FT4. TPO-Abs and Tg-Abs were measured by chemiluminescence immunoassays (LUMI test anti-TPO and LUMI test anti-thyroglobulin, respectively; Brahms, Berlin, Germany). Improved versions of both assays became available during follow-up: the detection limits of these new assays were 30 kU/L for TPO-Abs and 20 kU/L for Tg-Abs. The TPO-Ab concentrations obtained with the old assay were multiplied by a factor 0.72 to obtain comparative values in the new assay. TPO-Ab and Tg-Ab concentrations were considered to be positive at values greater than 100 kU/L.

Serum growth factors

We studied a panel of five growth factors at baseline: SCF, IGFBP-2, EGF, BDNF and IL-7. Serum concentrations were measured using the bead-based Luminex system. These multiplexed sandwich immunoassays were developed from commercially available capture and detection antibodies (R&D systems) and standard proteins, validated and approved at Myriad-EDI-GmbH (Reutlingen, Germany) according to methods described previously (24). Subject and healthy control samples were run singular. Assays were measured on either the Luminex FlexMap-3D or Luminex 200 system. Results are expressed as picograms per ml or nanograms per ml.

EGF, IL-7, SCF, BDNF were measureable in all samples. For IGFBP-2 6.6% of the values were above the detection limit. Values above the detection limit were set to the highest value observed (IGFBP-2: 19666.5 ng/ml).

Statistics

Test assumptions were checked by plotting of the data and depending on the distribution pattern, parametric (Student's T-test) or nonparametric group comparisons (Mann-Whitney U test) were used for unadjusted group comparisons. All analyses comparing HC, SC and NSC groups were subsequently adjusted, for which we used ANOVA. We adjusted for smoking, the usage of oral contraceptives, age, BMI and FT4 levels. Because SCs and NSCs were matched for age, smoking and estrogen use we did not adjust for these factors when comparing SCs and NSCs. If necessary we transformed dependent variables by the natural logarithm. In order to improve the interpretability of the group estimates, data are expressed as median with 95%

confidence intervals which were calculated using a bootstrap procedure with 1000 draws. For IGFBP-2, residuals of the regression analyses remained non-normal after transformation due to outliers (n=8), however, we can reliably report the outcomes of the regression analyses as these were in line with unadjusted non-parametric results and also remained similar after exclusions of the outliers.

In addition a dendrogram was constructed by SPSS using hierarchical cluster analysis of the serum analytes using the between-groups linkage method. For this analysis, we selected analytes from the previous study that were significantly different between healthy controls and subjects or between NSCs and SCs (S1 Table) and were part of a cluster in that study, and combined them with the levels of growth and differentiation factors assessed in the current study (11). The associations between TSH, FT4 and the serum growth factors were analyzed by linear regression analyses. Level of significance was set at $P<0.05$ (2 tailed). Statistical analysis was performed using SPSS Statistics for Windows, version 21 (IBM Corp., Armonk, NY, USA).

Results

As a result of the matching procedure, SCs and NSCs were not different regarding age, current smoking behavior, Body Mass Index (BMI), current estrogen use and TSH or FT4 levels (Table 1). None of the subjects were using chronic medication. TSH levels were not associated with any of the growth and differentiation factors (data not shown). FT4 levels were positively associated with IGFBP-2 levels (β±SE 0.16 ±0.075; $P=0.037$) and we observed a non-significant trend with SCF (β±SE 1.2 ±0.68; $P=0.069$). FT4 was not associated with the other growth factors.

Table 1. Baseline characteristics

							P-values		
	Controls		**SC**		**NSC**		**SC vs C**	**NSC vs C**	**NSC vs SC**
Number of subjects	30		30		31				
Age, mean (range)	35.2	(21-61)	33.3	(18-61)	33.5	(19-62)	0.5	0.56	0.93
BMI, mean (range)	22.7	(18-33)	24.1	(19-41)	24.2	(19-42)	0.22	0.17	0.95
Current smoking, %	12	(40%)	14	(46%)	14	(45%)	0.61	0.69	0.9
Current estrogen use, %	5	(17%)	11	(37%)	12	(38%)	0.055	0.083	0.87
TSH median (95% CI)	1.3	(1.1-1.7)	1.4	(1.3-1.7)	1.2	(1-1.5)	0.36	0.66	0.13
FT4 median (95% CI)	13.2	(12.9-14.6)	12.8	(12.4-13.5)	13.7	(13-14.2)	0.53	0.60	0.25

Characteristics of healthy controls (C) and relatives of AITD patients grouped for TPO antibody conversion during follow-up (seroconverters (SC) and non seroconverters (NSC)). Due to a lack of serum, the total number of subjects per group is not equal to the original 32.

Subjects versus healthy controls

Table 2 shows the median levels of the 5 tested growth and differentiation factors in the healthy controls and in the subjects. IGFBP-2 levels were significantly higher in the relatives than in the healthy controls (P=0.028). Serum levels of EGF were also significantly higher in the relatives than in the healthy controls (P=0.003). Serum levels of BDNF were significantly lower in the relatives than in the healthy controls (P<0.001). Serum levels of SCF and IL-7 were not statistically different between relatives and healthy controls.

Table 2. Comparison of growth and differentiation factors between healthy controls and subjects

	Controls	Subjects	*P*-value	Adjusted *P*-value
EGF (pg/ml)	307.6 (110-409)	506.9 (428-612)	0.001	0.003
BDNF (ng/ml)	18.9 (14.6-22.7)	8.2 (7.3-9.3)	<0.001	<0.001
IGFBP-2 (ng/ml)	177.8 (142- 235)	252.5 (177-351)	0.073	0.028
SCF (pg/ml)	22.6 (17.4-26.4)	22.6 (16.7-27.1)	0.58	0.70
IL-7 (pg/ml)	4.0 (2.8-5.1)	3.7 (3.0-3.8)	0.18	0.26

Median levels (95% CI) of EGF, BDNF, IGFBP-2, SCF and IL-7 in healthy controls and in subjects overall. Adjusted P-values are adjusted for age, BMI, smoking, oral contraceptive usage and FT4 levels.

Table 3. Serum levels of growth and differentiation factors in healthy controls (C), Seroconverting (SC) and Non-Seroconverting (NSC) family members.

				Adjusted *P*-values		
	Controls	SC	NSC	SC vs C	NSC vs C	SC vs NSC
EGF (pg/ml)	307.6 (110-409)	564.2 (411-803)	470.6 (355-612)	0.017	0.011	0.79
BDNF (ng/ml)	18.9 (14.6-22.7)	8.7 (5.1-10.8)	8.1(6.9-9.3)	<0.001	<0.001	0.45
IGFBP-2 (ng/ml)	177.8 (142- 235)	233.2 (153-376)	252.5 (164-390)	0.16	0.019	0.31
SCF (pg/ml)	22.6 (17.4-26.4)	26.5 (22.5-31.9)	16.7 (12.3-22.8)	0.49	0.26	0.017
IL-7 (pg/ml)	4.0 (2.8-5.1)	3.4 (2.7-3.8)	3.7 (3.0-4.0)	0.076	0.55	0.25

Median levels (95% CI) of EGF, BDNF, IGFBP-2, SCF and IL-7.

Seroconverters versus non-seroconverters

Table 3 shows that SC subjects had significantly higher levels of SCF than NSC subjects (P=0.017). Serum levels of SCF were not statistically different between SCs and healthy controls and between NSCs and healthy controls (P=0.49 and P=0.26, respectively). The serum levels of IGFBP-2, EGF, BDNF and IL-7 were not significantly different between SCs and NSCs. Serum levels of EGF were significantly higher in SCs and in NSCs than in healthy controls (P=0.017 and P=0.011, respectively). Serum levels of BDNF were significantly lower in SCs and in NSCs than in healthy controls (P<0.001 in both groups). A non-significant trend

towards lower levels of IL-7 in the SCs than in controls was observed (*P*=0.076). IGFBP-2 levels were higher in both groups of subjects than in healthy controls which was significant in the NSCs (*P*=0.019).

Cluster analysis

Taking the analytes which we have previously determined also into account we found in the cluster analysis two mutually correlating clusters of analytes: one stronger inter correlating cluster A and a weaker inter correlating cluster B (Figure 1) (11). Cluster A contained the inflammatory cytokines/chemokines IL-1β, CCL3 and IL-6, the connective tissue modulator MMP-13 and the hematopoietic/neuronal growth and differentiation factor SCF, while cluster B contained the pro-inflammatory chemokines CCL2 and CCL4, the endothelial adhesion molecule sVCAM-1, PDGF-BB, and the T cell and NK cell growth factor IL-7. The heat map shows that the growth factors IGFBP-2, EGF and BDNF barely correlated to each other and to the other analytes.

Table 4. Patterns of expression levels of cytokines, chemokines and growth and differentiation factors, assessed in the previous study of Beumer *et al.* and in the current study [7].

	NSC vs HC	SC vs NSC	SC vs HC
Pattern 1			
Fibronectin (µg/ml)	↑↑	≈	↑↑
IGFBP-2 (ng/ml)	↑↑	≈	≈
EGF (pg/ml)	↑↑	≈	↑↑
Pattern 2			
CCL4 (pg/ml)	↓↓	≈	↓↓
MMP-13 (ng/ml)	↓↓	≈	↓↓
CCL2 (pg/ml)	↓↓	≈	≈
sVCAM-1 (µg/ml)	↓↓	≈	↓↓
PDGF-BB (pg/ml)	↓↓	≈	↓↓
BDNF (ng/ml)	↓↓	≈	↓↓
Pattern 3			
IL-1β (pg/ml)	↓↓	↑↑	≈
IL-6 (pg/ml)	↓↓	↑↑	≈
CCL3 (pg/ml)	↓↓	↑↑	≈
SCF (pg/ml)	≈	↑↑	≈
Other			
IL-7 (pg/ml)	≈	≈	≈

Patterns of expression levels in serum compounds between seroconverters (SC) and non-seroconverters (NSC) and healthy controls (HC). ↑↑ and ↓↓ indicates significantly higher or lower serum levels respectively, and ≈ indicates that serum levels are not significantly different (S1 table).

Regarding the pattern of expression in the two study groups (SCs and NSCs) three patterns of reactivity could be detected (S1 Table and Table 4). There were factors, which were higher in the serum of both SCs and NSCs than in controls, such as the growth factors IGFBP-2, EGF and the repair factor Fibronectin. There were also factors that were lower in the serum of both SCs and NSCs than in controls, such as CCL4, CCL2, sVCAM-1, BDNF and PDGF-BB. Finally, there were factors that were higher in the SCs than in the NSCs such as IL1-β, IL-6, CCL3 and SCF (Supplemental Table 1 and Table 4).

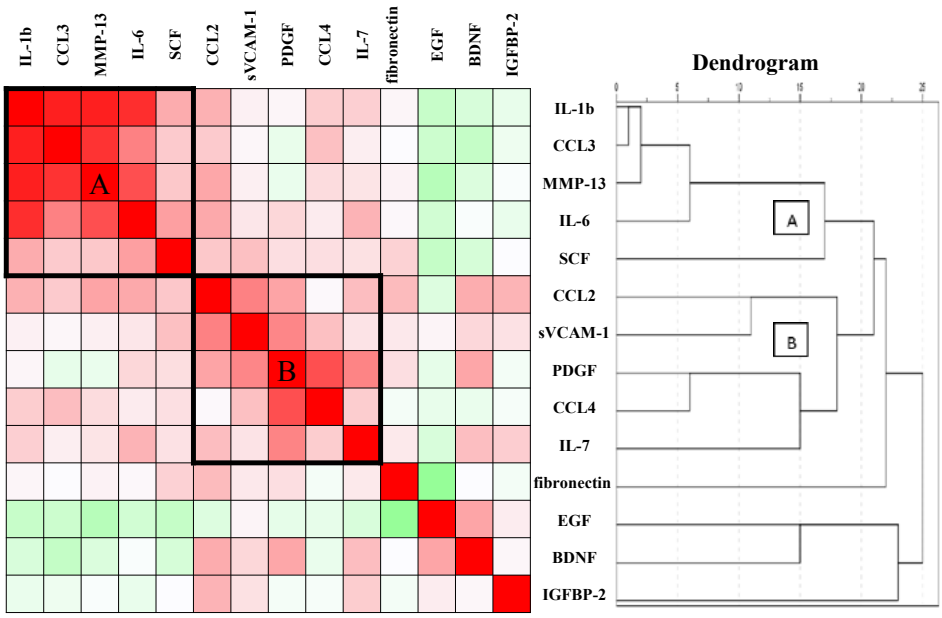

Figure 1. Cluster analysis. Heat map of hierarchical cluster analysis of the serum levels of cytokines, chemokines, growth factors and tissue remodeling factors in the relatives. Color-coded correlation matrix illustrates Pearson's correlation coefficients. Significant positive correlations are given in the red scale (darkest red are correlation coefficients > 0.50), and significant negative correlations are given in the (dark) green scale. Lighter fields are not significant. In addition, a dendrogram is presented as a result of the hierarchical clustering. A indicates cluster A and B indicates cluster B.

Discussion

The present study shows that euthyroid females, who are relatives of AITD patients and at risk of developing AITD, have an aberrant serum level of 4 of the 5 tested hematopoietic/neuronal growth and differentiation factors, i.e. of BDNF, IGFBP-2, EGF and SCF. BDNF levels were significantly lower and IGFBP-2 and EGF higher expressed in sera of the relatives of the AITD patients (in both SCs and NSCs) than in healthy controls. IL-7 levels were normal. We also found in the healthy relatives, who converted in the following 5 years to TPO-Ab positivity, significantly higher serum levels of SCF than in relatives who did not.

It is of note that the 5 studied factors have been highlighted as serum biomarkers for major mood disorders in several studies (19-23) and are involved in neurogenesis, neuroprotection and hematopoietic differentiation (25-29). This is in particular known for BDNF. Neurotrophic factors, like BDNF, play an important role in neuronal plasticity, modulating not only axonal and dendritic growth and remodeling, but also neurotransmitter release and synapse formation (30, 31). Neuronal plasticity is a complex process which is illustrated by the complex interaction between the neurotrophic factors and its receptors. The cellular actions of BDNF, for example, are mediated through TrkB (tyrosine kinase receptor) and by p75 neurotrophin receptor ($p75^{NTR}$) (32). Binding of dimeric BDNF causes dimerization of TrkB receptor and autophosphorylation of intracellular tyrosine residues. Activation of TrkB receptor leads to signaling cascades involving activation of Ras/ERK pathway, phosphatidylinositol 3-kinase (PI3K) and Phospholipase Cγ (33). The Ras pathway regulates neuronal survival and differentiation through downstream signaling that includes c-RAF/B-Raf/ERK1/ERK2. Brunoni *et al.* showed in a meta-analysis that BDNF levels were decreased in patients with a major depressive disorder and were also associated with clinical changes in depression (34).
This study also extends our previous study and shows that the here reported 5 growth factors and the previously reported 9 growth factor/cytokines form three patterns of reactivity in the relatives of AITD patients when compared to healthy subjects and depending on TPO-Ab seroconversion within the next 5 years (11). This study and the previous one therefore underscore the widespread changes in immune-neuro-endocrine molecular networks that apparently precede the appearance of TPO-Abs, which opens avenues for developing assays for the detection of individuals at risk for thyroid autoimmunity.
Combining this study with the previous one we found factors which were raised in the relatives, irrespective of later seroconversion, such as the growth factors IGFBP-2, EGF and the repair factor Fibronectin. None of these factors inter correlated. There were also factors that were reduced in the serum of the relatives irrespective of later seroconversion, such as the chemokines CCL4, CCL2, the adhesion molecule sVCAM-1, and the growth factors BDNF and PDGF-BB. Many of these factors inter correlated in cluster B in the dendrogram constructed in this article. Taken these two patterns of reactivity together, the serum aberrancies suggest another state of growth regulation of multiple cell lines (including neuronal and hematopoietic cells) and another state of leukocyte migration in relatives of AITD patients.
Finally, there were factors that were higher in the SCs than in the NSCs such as IL1-β, IL-6, CCL3 and SCF, while often being lower in the NSCs than in controls. These factors correlated to each other in cluster A in the dendrogram. We assume that the generally low expression in NSCs in cluster A reflects an immune suppressive state preventing autoimmunity, while a rise of these pro-inflammatory compounds precedes a conversion to TPO-Ab positivity and thus may reflect a very early stage of thyroid auto reactivity. In recent studies, the importance of T helper 1 lymphocytes in the induction of such auto-inflammatory state has been shown (35). A

further investigation of cytokines and chemokines reflecting the higher state of the T helper 1 system (such as IL-12, IFN-γ and CXCL-10) in relatives of AITD index cases deserves further attention (36, 37).

A limitation of our study is the relatively small sample size. Also, because this is an explorative study which focused on 5 non-random selected analytes, we did neither take type I errors into account nor applied a correction for multiple testing. Our study is also limited by the fact that we did not assess the association between the levels of these growth and differentiation factors and the mood state of the relatives (sampling of the sera occurred 15 years ago, at the time we were unaware of the link between AITD and depression). Next, we used a BDNF antibody in our immunoassay, which was developed to measure mature BDNF. It is now known that there are other assays and antibodies on the market that also measure pro-BDNF (38). It has been reported, however, that in particular mature BDNF is important in major depression (39) although this needs exploration. Furthermore, also other factors important in neuronal growth and differentiation should be taken into account, such as glial-cell-line-derived neurotrophic factor (GDNF). GDNF signals through a multicomponent receptor complex comprising the Ret proto-oncogene (RET) tyrosine kinase and the GDNF family receptor (GFR)α (40). Following GDNF binding in the presence of co-receptor GFRα, RET becomes dimerized and tyrosine phosphorylated and triggers different pathways (Ras-MAPK, PI3K-Akt). Finally, in the paragraph above, we have highlighted that other immune factors which are linked to the induction of thyroid autoimmunity (such as T helper 1 related factors) should be explored. It is also evident that our studies need confirmation and expansion in larger families and follow-up studies, taking many more neuronal, endocrine and immune factors into consideration to unravel the changes in immune-neuro-endocrine molecular networks that precede and probably underlie the development of AITD and mood disorders.

Since a large number of autoimmune hypothyroid patients have a diminished cognitive and psychological function despite adequate levothyroxine replacement therapy, another next step could be to measure the growth and differentiation factors in these patients and to assess the association with these symptoms.

We conclude that subjects at risk for AITD show changes in growth and differentiation factors in serum, which are both active as neuronal and hematopoietic growth and differentiation factors and are abnormally expressed in patients with mood disorders. This suggests that shared growth and differentiation defects in both the hematopoietic and neuronal system may underlie both thyroid autoimmunity and mood disorders.

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Supplemental Table. Serum levels of cytokines, chemokines and growth factors of healthy controls (HC), Non-Seroconverting (NSC) and Seroconverting (SC) relatives assessed in the previous study and in the current study, grouped according to patterns of expression.

	Pattern	Cluster	HC		NSC		SC		NSC vs HC	SC vs NSC	SC vs HC
			M	*SE*	*M*	*SE*	*M*	*SE*	*P value*	*P value*	*P value*
Fibronectin (µg/ml)	1		367.1	(47.5)	646.2	(33.6)	590.6	(28.4)	**<0.001**	0.21	**<0.001**
IGFBP-2 (ng/ml)	1		848.1	(649.2)	3410.4	(1302)	1552.1	(899.4)	**0.019**	0.20	0.16
EGF (pg/ml)	1		314.6	(43.4)	598.1	(70.1)	637.0	(86.1)	**0.011**	0.80	**0.017**
CCL4 (pg/ml)	2	B	77.8	(25.5)	23.4	(18.6)	6.9	(5.7)	**<0.001**	0.13	**<0.001**
CCL2 (pg/ml)	2	B	474.4	(31.7)	191.7	(28.1)	166.6	(24.4)	**<0.001**	0.32	0.271
sVCAM-1 (ng/ml)	2	B	1246.8	(98.9)	481.1	(39.7)	491.0	(37.1)	**<0.001**	0.93	**<0.001**
PDGF-BB (pg/ml)	2	B	2470	(364.5)	1145.7	(72.0)	1017.7	(47.9)	**<0.001**	0.12	**<0.001**
BDNF (ng/ml)	2		17.9	(1.7)	8.7	(0.7)	7.9	(0.7)	**<0.001**	0.45	**<0.001**
MMP-13 (ng/ml)	2	A	2.2	(0.6)	0.62	(0.1)	1.1	(0.3)	**<0.001**	0.27	**0.001**
IL-1B (pg/ml)	3	A	269.3	(89.1)	72.2	(48.1)	564.2	(318.8)	0.004	**0.012**	0.82
IL-6 (pg/ml)	3	A	10.5	(5.7)	1.8	(0.7)	20.3	(13.5)	0.011	**0.041**	0.47
CCL3 (pg/ml)	3	A	631.6	(177.6)	209.9	(67.1)	1672.6	(1036.6)	0.012	**0.009**	0.74
SCF (pg/ml)	3	A	24.9	(2.3)	20.0	(2.3)	26.7	(2.3)	0.26	**0.017**	0.49
IL-7 (pg/ml)			5.3	(1.0)	3.9	(0.3)	3.3	(0.3)	0.23	0.55	0.076

Chapter 5

Thyroid Hormone and its Metabolites in Relation to Quality of Life in Patients Treated for Differentiated Thyroid Cancer

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Abstract

Background: Levothyroxine (LT4) is the standard of care in patients with hypothyroidism. Despite this replacement therapy, quality of life (QoL) remains impaired in a substantial amount of patients. The reasons for this are still a matter of debate. Suggested causes include lack of endogenous T3 secretion by the thyroid, changes in other thyroid hormone metabolites, and interference by autoimmune processes.

Objective: To investigate the association between thyroid function tests (TFTs) and QoL in patients with a history of differentiated thyroid cancer on LT4 monotherapy. These patients lack endogenous thyroidal T3 secretion in the absence of autoimmune disease. Materials and Methods: This is a cross-sectional study in 143 patients (69.2% female). Initial therapy consisted of total thyroidectomy followed by radioiodine ablation minimally one year before inclusion. We assessed health-related QoL (RAND-36), thyroid specific QoL (ThyPRO) and fatigue with the Multidimensional Fatigue Inventory. Extensive TFTs were assessed, including 3,5-diiodo-L-thyronine (3,5-T2).

Results: Mean age was 50.2 years and mean time since diagnosis was 8.4 years. Median TSH was 0.042 mU/l, total T4 145.0 nmol/l, free T4 25.6 pmol/l, total T3 1.93 nmol/l, reverse T3 0.53 nmol/l, and 3,5-T2 0.86 nmol/l. Multiple linear regression analyses did not show any association between QoL and the different TFTs, including T4/T3 and 3,5-T2/T3 ratios reflecting peripheral metabolism.

Conclusion: We did not find any association between TFTs and QoL in athyreotic patients on LT4 monotherapy. Our data do not provide evidence that a slight increase in dose improves fatigue or well-being in hypothyroid patients on LT4 therapy.

Introduction

Hypothyroidism is one of the most common endocrine disorders(1). Despite adequate restoration of biochemical euthyroidism with replacement therapy with levothyroxine (LT4), a substantial part of patients (~10-15%) display significant impairment of psychological well-being compared to controls of similar age and sex and show decrements in health status (2-6). There are a couple of explanations for these persisting symptoms. First, the presence of associated autoimmune diseases or thyroid autoimmunity per se could account for persisting symptoms (7-9). However, cured patients with differentiated thyroid carcinoma (DTC) on LT4 replacement therapy have also been shown to have an impaired quality of life (QoL) [10],(11). Second, it might be the inadequacy of LT4 treatment to restore physiological T4 and T3 concentrations in serum and tissues (12, 13). Approximately 15% of circulating T3 in euthyroid patients is secreted by their thyroid gland. Nevertheless, in patients with hypothyroidism, LT4 monotherapy is the standard of care. The peripheral conversion of LT4 to T3 makes it possible to achieve normal T3 concentrations in patients treated with LT4, albeit with the necessity for maintaining T4 concentrations at the higher end of the normal range (14-16). Studies in hypothyroid rats showed that LT4 monotherapy was not able to normalize concentrations of T4 and T3 in all tissues (17). The LT4 dose needed to normalize thyroid hormone concentrations was different for each tissue, and supra- physiological serum T4 concentrations had to be reached in most tissues to normalize their T3 concentrations. However, addition of synthetic T3 to LT4 treatment regimens is controversial. Although two studies found benefits of adding T3 therapy (18),(19), most studies did not (16, 20). A meta-analysis of 11 studies that had been performed so far comparing the effectiveness of T4-T3 combination therapy vs LT4 monotherapy, did not find any improvement of symptoms and QoL for T4-T3 combination therapy (21).

Studies investigating the relationship between TFTs and QoL have not produced unequivocal results. Importantly, most studies were performed in patients with autoimmune thyroid disease (AITD) in which autoimmunity might have affected QoL (2, 3, 6). Anecdotal evidence suggests that some patients with hypothyroidism benefit from titrating the LT4 dosage until serum TSH is in the lower part of the reference range although this was not confirmed by the only clinical trial investigating this approach (22).

Furthermore, it remains unknown whether other endogenous thyroid hormone metabolites with T3-agonistic or antagonistic activities, like 3,5-diiodo-L-thyronine (3,5-T2), might play a role in the persisting symptoms and decreased QoL among hypothyroid patients. Recent studies have shown that 3,5-T2 concentrations are higher in thyroidectomized patients on LT4 substitution therapy as compared with healthy controls, suggesting extrathyroidal production or conversion (23). The metabolic pathways or enzymes involved in this reaction in vivo have not been characterized, yet growing evidence shows that 3,5-T2 plays an important role in energy metabolism (24),(25). The association between 3,5-T2 and QoL amongst hypothyroid patients has never been investigated.

In this cross-sectional study we therefore measured classical TFTs and assessed concentrations of 3,5-T2 with a recently developed competitive chemiluminescence immunoassay (23). We subsequently investigated whether thyroid hormones or their metabolites were associated with various QoL outcomes in patients with a history of DTC, lacking functional thyroid tissue but who were not affected by AITD.

Patients and Methods

DTC patients, 18-80 yrs. old, were consecutively recruited from the outpatient clinic of the Erasmus Medical Center, between November 1st 2013, and December 31st 2014. Initial therapy consisted of total thyroidectomy, followed by ablation therapy with I-131 (except for the patients with medullary thyroid carcinoma; N=7). Patients were eligible for inclusion in the study if either cured from DTC and initial therapy was finished at least one year before study inclusion or if they had stable disease; TSH concentrations were lower than 4.3 mU/l and no changes were made in the LT4 dosage during the past 10 weeks (to ensure that all patients were adequately treated with LT4 monotherapy in a stable dosage); patients were able to comprehend and complete the Dutch questionnaires; and had negative titers for thyroglobulin antibodies (Tg-Abs< 40 IU/ml). Patients with other malignancies or an active inflammatory disease were excluded.

In total 263 patients were screened for eligibility. Out of the 159 patients meeting the inclusion criteria, 5 patients refused participation. Of the 154 enrolled patients, 11 did not complete the questionnaire.

Cure after initial therapy was defined as: a negative whole body iodine 131 post-therapy scan, a Tg concentration less than 0.9 µg/l on LT4 therapy in absence of Tg-Abs (<10 IU/ml), or a calcitonin concentration of less than 2 ng/l for patients with MTC, an undetectable Tg concentration after stimulation with recombinant TSH, and no signs of tumor remnant/ recurrence on the ultrasound of the neck.

The study protocol was approved by the Medical Ethics Committee of the Erasmus Medical Center and written informed consent was obtained from all study participants.

Quality of life questionnaires

Thyroid-specific patient reported outcome measure (ThyPRO)

The ThyPRO is a thyroid-specific QoL questionnaire which consists of 85 questions summarized in 13 scales. It detects clinically relevant differences among thyroid patients and has good cross-cultural validity [25,(26)]. Scores vary from 0-100 with higher scores indicating more thyroid-related complaints. Since the Goiter symptoms scale, the Eye symptoms scale, and the Cosmetic complaints scale are not relevant in patients with treated thyroid carcinoma, these three scales were not analyzed.

Multidimensional fatigue index-20 (MFI-20)

The MFI is a 20-item self-report instrument designed to assess fatigue. Scores vary from 0 to 20 with higher scores indicating greater fatigue. It covers the following dimensions: General Fatigue, Physical Fatigue, Mental Fatigue, Reduced Motivation and Reduced Activity (27).

RAND-36-item health survey (RAND-36)

The RAND-36 is the validated Dutch version of the SF-36. It is a generic questionnaire which determines the overall health-related QoL during the previous 30 days (28). It consists of 9 subscales: physical functioning, social functioning, role limitations because of physical health problems, role limitations because of emotional health, general mental health, vitality, bodily pain, general health perception and general health change. Scores are expressed on a 0-100 scale, and higher scores are associated with a better QoL. Two well-known summary scores of the subscales were computed: the mental component score and the physical component score (29). The scores on the RAND-36 subscales were compared with an age-matched Dutch reference group(28). Reference values were computed from a random sample of 1063 persons (65% females) from the register of the city of Emmen.

Thyroid function measurements

Peripheral blood samples were obtained from all participants. Serum Free T4 (FT4, reference range 11-25 pmol/l), total T4 (TT4, reference range 58-128 nmol/l) and total T3 (TT3, 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 (reference range 0.4-4.3 mU/l) was determined by immunometric assay (Immulite 2000 XPi, Siemens, Den Haag, the Netherlands) and rT3 concentrations (reference range 0.22-0.54 nmol/l) were analyzed by radioimmunoassay (ZenTech, Angleur, Belgium). Serum 3,5-T2 concentrations were quantified with a recently developed monoclonal antibody-based chemiluminescence immunoassay in the laboratory of the Institute of Experimental Endocrinology, Charité-Universitätsmedizin Berlin in Germany (23). In 761 euthyroid participants of the population-based Study of Health in Pomerania median 3,5-T2 concentrations were 0.24 nmol/l (1st quartile: 0.20 nmol/l; 3rd quartile: 0.37 nmol/l) (30).

Statistical analysis

The association between TFTs and patient characteristics was analyzed by linear regression analyses. Nonlinearity was assessed using restricted cubic splines analyses, or sensitivity analyses focusing on variations in R-squared values in which independent variables were added to the model with quadratic or cubic terms and multiple linear regression models were built accordingly. TSH, 3,5-T2, TT3, TT4 and rT3 showed non-normally distributed residuals

and were logarithmically transformed to normalize the skewed distribution. The association between QoL and TFTs was analyzed by linear regression analyses as well. All analyses were adjusted for age, sex, body mass index (BMI), the number of years since diagnosis, the number of drugs used and being cured or not. The number of drugs was used as a proxy variable for general health and/or comorbidities. The mental component scores, the physical component scores, some of the RAND-36 subscales and the MFI scales showed non-normal distribution of the residuals and had to be normalized using a Tukey transformation. As transformation did not normalize the residuals of ThyPRO scales, we used a logistic regression analyses investigating the risk of being in the highest tertile for symptom score. In addition, we also investigated the association between TFTs and ThyPRO scales by ordinal logistic regression (with tertiles of the symptom scores) and standard linear regression which all yielded similar results (data not shown). The health-related QoL (RAND-36) scores were compared with a Dutch reference group using the non-parametric
one sample sign test. In order to cope with the large number of statistical tests within our study population we used the Benjamini-Hochberg false discovery rate controlling procedure and we allowed for a maximum of one result of all non-overlapping tests (i.e. not counting ratios and not counting multiple fatigue measurements) to be false positive; this corresponded with a cut-off of $P < 0.012$ for statistical significance. Statistical analysis was performed using SPSS Statistics for Windows, version 21 (IBM Corp., Armonk, NY, USA) and R statistical software v.3.03 using the rms and fdrtool package.

Results

Characteristics of the study patients are shown in Table 1. We analyzed 143 patients with a mean age of 50.2 years of whom the vast majority (82.6%) was diagnosed with a papillary thyroid carcinoma. All patients had undergone a total thyroidectomy. A lymph node dissection was performed in 35 % of the patients, mostly a combined central and ipsilateral procedure. Eighty percent of patients were cured and the others had stable disease.

TFTs

At the time of the survey, median TSH concentrations were 0.042 mU/l (Inter Quartile Range 0.007-0.21), median TT4 concentrations were 145.0 nmol/l (128.0-170.0), median FT4 concentrations were 25.6 pmol/l (23.0-29.0), median rT3 concentrations were 0.53 nmol/l (0.45-0.65), while median TT3 concentrations were 1.91 nmol/l (1.74-2.10) (Table 1). Median 3,5-T2 concentrations were 0.86 nmol/l (0.55-1.17), which is higher than the median 3,5-T2 concentrations assessed in serum from thyroidectomized and LT4-substituted patients (0.48 ± SD 0.03 nmol/l, n=100) by Lehmphul et al.(23).

Table 1. Characteristics of study participants

Male	44 (30.8)
Female	99 (69.2)
Age, mean ±SD (range), years	50.2 ± 13.3 (19.4-78.4)
BMI, mean ±SD, kg/m2	27.9 ± 5.6
Time since diagnosis, mean ±SD, years	8.4 ± 7.8
Dose LT4, mean ±SD, µg	180.9 ± 55.0
Dose LT4 per kg, mean ±SD, µg/ kg	2.18 ± 0.54
Number of drugs, mean ±SD (range)	2.8 ± 2.3 (1-11)
Diagnosis *N (%)*	
PTC	118 (82.5)
FTC	18 (12.6)
MTC	7 (4.9)
Cured	114 (79.7)
Surgery *N (%)*	
Total thyroidectomy	93 (65)
Total thyroidectomy + neck dissection	50 (35)
Postoperative hypoparathyroidism *N (%)*	20 (14)
Number of I-131 therapies *N (%)*	
0 (MTC)	7 (4.9)
1	71 (49.7)
2	30 (21.0)
3	22 (15.4)
4	7 (4.9)
5	2 (1.4)
7	1 (0.7)
Unknown	3 (2.1)
TSH, median (reference range), mU/l	0.042 (0.4-4.3)
FT4, median (reference range), pmol/l	25.6 (11.0-25.0)
TT4, median (reference range), nmol/l	145.0 (58.0-128.0)
TT3, median (reference range), nmol/l	1.93 (1.4-2.5)
rT3, median (reference range), nmol/l	0.53 (0.22-0.54)
3,5-T2, median (IQR), nmol/l	0.86 (0.55-1.17)

The dosage of LT4 per kilogram was positively associated with FT4 and TT4 concentrations (P= 0.003 and P= 0.004 respectively) while there was a trend towards a negative association with TSH concentrations (P= 0.017). In contrast, TT3, rT3 and 3,5-T2 concentrations were not associated with the dosage of LT4 per kilogram (Table 2). In this cohort, 3,5-T2 concentrations showed a trend towards a positive association with age (P= 0.04), but no association was noted for BMI or sex of the subjects. TT4 concentrations tended to be lower in males (P= 0.017) and TT4 concentrations were positively associated with BMI of the subjects (P< 0.001). FT4 concentrations were associated with TSH, TT3 and rT3 concentrations but not with 3,5-T2 concentrations (Figure 1).

Table 2. Association of patient characteristics with TFTs

	TSH		**FT4**		**TT4**		**TT3**		**rT3**		**3,5-T2**	
	β ±SE	*P*	β ±SE	*P*	β ±SE	*P*	β ± SE	*P*	β ±SE	*P*	β ±SE	*P*
Age	0.013 ± 0.01	0.39	0.05 ± 0.03	0.12	-0.001 ± 0.001	0.24	-0.002 ± 0.001	0.015	-0.003 ± 0.002	0.18	0.007 ± 0.004	0.04
BMI	0.007 ± 0.03	0.83	0.06 ± 0.07	0.46	0.014 ± 0.03	**<0.001**	0.004 ± 0.002	0.07	0.008 ± 0.004	0.047	-0.008 ± 0.008	0.31
Sex	-0.33 ± 0.40	0.41	-1.2 ± 0.90	0.20	-0.09 ± 0.035	0.017	0.03 ± 0.03	0.25	0.06 ± 0.060	0.52	-0.001 ± 0.096	0.99
Dosage/ kg	-0.87 ± 0.36	0.017	2.45 ± 0.82	**0.003**	0.094 ± 0.03	**0.004**	0.04 ± 0.03	0.09	0.04 ± 0.060	0.53	-0.04 ± 0.09	0.66

Results of the multiple linear regression analysis of the association between patient characteristics and TFTs. The regression coefficients (β) with standard errors (SE) are shown in the table.

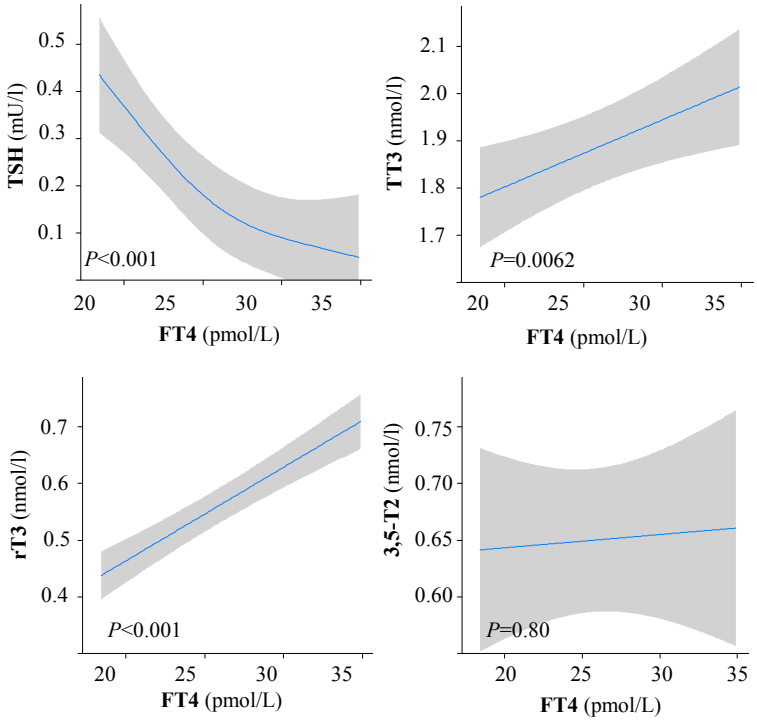

Figure 1. The association between FT4 and TSH, TT3, rT3 and 3,5-T2.
Analysis were adjusted for BMI, age and sex of the subjects.

Table 3. Comparison of RAND-36 scores with the Dutch population.

RAND-36 Subscales	Study Patients	Reference Scores	*P*
Physical functioning	85.0 (70.0-95.0)	79.9 (72.7-90.0)	0.35
Social functioning	75.0 (62.5-100.0)	86.4 (86.1-88.0)	<0.001
Role limitations; physical	68.75 (50.0-93.8)	78.9 (76.5-82.9)	<0.001
Role limitations; emotional	75.0 (58.3-100.0)	83.6 (82.2-86.8)	<0.001
General mental health	65.0 (55.0-80.0)	76.9 (76.7-77.1)	<0.001
Vitality	50.0 (37.5-62.5)	67.1 (67.0-67.5)	<0.001
Bodily pain	100.0 (67.3-100.0)	80.5 (74.7-83.8)	0.008
General health perception	60.0 (40.0-70.0)	71.6 (64.4-74.0)	<0.001
General health change	50.0 (50.0-50.0)	51.9 (48.7-55.4)	0.001

RAND-36 scores in study patients compared with age-adjusted reference values from the Dutch population. Data are expressed as median (IQR).

Table 4. Association between TFTs and component scores of the RAND-36 and most relevant ThyPRO scales

	RAND-36				**ThyPRO**							
	Mental component score		**Physical component score**		**Hyperthyroid symptoms**		**Tiredness**		**Cognitive problems**		**Depressivity**	
	B ± SE	*P*	B ± SE	*P*	OR (95%CI)	*P*	OR (95%CI)	*P*	OR (95%CI)	*P*	OR (95%CI)	*P*
TSH	0.11 ± 0.13	0.39	0.05 ± 0.12	0.67	0.69 (0.35-1.4)	0.30	0.44 (0.7-2.5)	0.44	0.9 (0.5-1.7)	0.72	0.96 (0.5-1.8)	0.96
FT4	0.013 ± 0.016	0.43	-0.003 ±0.015	0.86	1.01 (0.9-1.1)	0.78	0.99 (0.9-1.07)	0.75	1.01 (0.9-1.1)	0.86	0.97 (0.9-1.05)	0.40
TT4	0.002 ± 0.003	0.37	0.001 ± 0.002	0.57	0.99 (0.98-1.0)	0.49	0.99 (0.97-1.0)	0.99	0.99 (0.98-1.01)	0.22	0.99 (0.98-1.01)	0.09
TT3	0.18 ± 0.27	0.50	0.28 ± 0.24	0.25	1.57 (0.4-5.9)	0.50	0.38 (0.09-1.5)	0.17	0.38 (0.1-1.4)	0.15	0.53 (0.1-2.0)	0.34
rT3	1.16 ± 0.59	0.052	0.40 ± 0.54	0.46	0.13 (0.006-2.8)	0.19	0.39 (0.02-8.1)	0.55	2.1 (0.1-35.8)	0.60	0.18 (0.009-3.3)	0.25
3,5-T2	-0.02 ± 0.01	0.90	-0.30 ± 0.16	0.07	1.3 (0.5-3.3)	0.54	3.3 (1.2-9.0)	0.02	2.1 (0.8-5.4)	0.12	0.55 (0.2-1.4)	0.21
T2/T3 ratio	-0.11 ± 0.31	0.73	-1.8 ± 0.73	0.013*	1.18 (0.3-5.7)	0.84	7.1 (1.2-40.6)	0.029	3.6 (0.7-18.8)	0.12	0.38 (0.08-1.9)	0.24
T4/T3 ratio	0.004 ± 0.006	0.48	-0.003 ±0.005	0.55	0.98 (0.94-1.01)	0.16	1.01 (0.98-1.04)	0.51	0.99 (0.96-1.03)	0.79	1.0 (0.97-1.03)	0.95
Time since diagnosis	0.03 ± 0.01	**0.012**	0.02 ± 0.009	0.038	0.96 (0.9-1.02)	0.18	0.9 (0.9-1.01)	0.08	0.94 (0.9-0.99)	0.046	0.95 (0.9-1.01)	0.083
Number of drugs	-0.13 ± 0.04	**0.001**	-0.14 ± 0.03	**<0.001**	1.1 (0.9-1.3)	0.30	1.08 (0.9-1.3)	0.38	1.03 (0.86-1.2)	0.78	1.18 (0.99-1.4)	0.073
Cured or not	0.07 ± 0.20	0.72	-0.04 ± 0.18	0.83	2.56 (0.8-8.4)	0.12	4.17 (1.1- 15.8)	0.035	1.9 (0.7-5.5)	0.21	1.45 (0.5-4.0)	0.48
BMI	0.007 ± 0.01	0.62	-0.03 ± 0.01	0.040	1.02 (0.95-1.1)	0.55	1.03 (0.96-1.1)	0.37	0.99 (0.93-1.06)	0.78	0.99 (0.92-1.05)	0.66

Results of the multiple linear regression analysis of the association between TFTs and component scores of the RAND-36 and of the logistic regression analyses between TFTs and most relevant scales of the thyroid specific questionnaire: ThyPRO. The regression coefficients (β) and the odds ratios (OR) are shown in the table.*a quadratic term was added to the model.

Quality of Life

Table 3 shows that our subjects scored lower on all RAND-36 subscales compared with a Dutch reference group, except for the subscales physical functioning and bodily pain. Table 4 and Supplemental Table 1 show the association of TFTs with the mental component score and the physical component score of the RAND-36, and the ThyPRO scales. None of the TFTs was associated with QoL outcomes in our subjects. As for other determinants of QoL, the number of drugs used by the patient showed a negative association that was consistent for both of the RAND summary scales (P< 0.001 and P= 0.001). Also, time since diagnosis consistently showed a trend towards a positive association with both summary scales of the RAND-36 (P= 0.038 and P= 0.012). Curation, BMI and hypoparathyroidism (data not shown) were neither associated with RAND summary scales nor with ThyPRO scales.

Supplemental Table 2 shows that there is no association between TFTs and the MFI dimensions. The number of drugs used by the patient was positively associated with fatigue in four of the five subscales of the MFI. However, after correction for multiple testing this association was no longer significant except for the dimension physical fatigue (P= 0.006).

Discussion

In this cross-sectional study among athyreotic patients with a history of DTC, there was no consistent association of TFTs with health-related QoL. Since all our patients lack functional thyroid tissue due to surgery and radioiodine ablation therapy, and none of the patients had evidence for AITD, we were able to study the isolated effects of LT4 monotherapy in a very homogenous group.

Our patients were treated with relatively high dosages of LT4 leading to high but not thyrotoxic TT4 and FT4 concentrations while their total TT3 concentrations remained within the reference range. Previous studies already demonstrated a decreased ratio of TT3 to TT4 in patients after thyroidectomy receiving LT4 monotherapy [16, 31] ,(15). However, these authors did not assess the association with QoL. In the current study, there was no association between TFTs, including their ratios, and health-related QoL or thyroid specific QoL. As fatigue is common among patients with thyroid disease, we also used the MFI to assess fatigue. However, there was no association between TFTs and fatigue either. Subgroup analysis of patients with the lowest TT3 concentrations did not show a decreased QoL or increased fatigue either (data not shown). This is in line with data from Walsh et al., who reported that small changes in LT4 dosage did not lead to changes in hypothyroid symptoms, well-being or QoL (22). Other authors could not find an association between QoL and TSH concentrations (10, 32, 33). Taken together, these data suggest that there is no relationship between a large set of thyroid function parameters and complaints of fatigue or impaired well-being in hypothyroid patients on LT4 therapy.

We speculated that other T4-derived endogenous thyroid hormone metabolites like 3,5-T2 might be involved in eliciting persisting complaints and symptoms. Pharmacological application of 3,5-T2 in rodent models enhances energy metabolism by increasing lipid oxidation and mitochondrial activity, activates brown adipose tissue thermogenesis and prevents hepatic steatosis and insulin resistance (25, 34), albeit some of these effects remain controversial (35-37). However, little is known about the role of 3,5-T2 in human metabolism. Recently, an association between 3,5-T2 concentrations and serum fasting glucose concentrations and leptin concentrations was detected in healthy euthyroid humans (30), an observation which might be of interest in context of frequent complaints of weight gain associated with LT4 replacement therapy after thyroidectomy (38, 39). However, we could not find any association between 3,5-T2 concentrations and QoL as well. Of course, we cannot rule out that the thyroid gland of healthy individuals produces other thyroid hormone metabolites, we are not able to assess yet. Such metabolites might be associated with QoL or might directly impact on or be associated with fatigue or other symptoms of discontentedness with LT4 therapy. Concentrations of 3,5-T2 were not associated with the dosage of LT4 and showed no association with (F)T4 and TT3 concentrations either.

Health-related QoL was overall lower in our patients compared with a Dutch reference group. This might be explained by the fact that our patients had been diagnosed with thyroid cancer(10, 11). However, health-related QoL and cancer disease status (being cured or not) was not correlated in our patients. Although health-related QoL and years since diagnosis showed some relation, this failed to reach significance after correction for multiple testing. However, since similar results have been reported for duration of cure in other cohorts of DTC patients(10), QoL seems to improve when time since diagnosis increases. A limitation of the reference group is that its characteristics are somewhat different. The reference group has a slightly lower percentage of females (65% vs. 69% in our cohort), was from another, more rural area in The Netherlands and questionnaires were taken in the 90's. The number of drugs used by the patient was negatively associated with QoL in the current cohort, which is most likely explained by co-occurrence of co-morbidities. Permanent hypoparathyroidism was not associated with QoL. BMI was not associated with QoL, which is in contrast with recent findings from Kelderman-Bolk et al., who found an association between QoL and BMI in hypothyroid patients, mostly caused by AITD (6). This might be explained by a difference in BMI between the two studies, since mean BMI was higher in their study patients than in our patients (29.1 kg/m^2 vs 27.9 kg/m^2, respectively).

This study is to our knowledge the first study that describes an extensive thyroid hormone metabolite profile, including rT3 and 3,5-T2 concentrations, in a population of athyreotic patients treated with LT4 in the context of an in depth QoL evaluation. This study has some limitations: first of all, the observational and cross-sectional design of the study limits the determination of causal inference, and second, we were only powered to statistically detect

relatively large effects due to the limited number of included participants and the well-recognized measurement error of QoL questionnaires. With regards to the latter, it is important to note that our results for QoL were consistent across different questionnaires and their subsets. It is therefore unlikely that the association between QoL and TFTs would change with increasing study participants. However, the questionnaires we used might have been not sensitive enough to detect an association with TFTs. The ThyPRO has been developed to measure the impact of any benign thyroid disease on health-related QoL, while our patients have been treated (and cured in majority of cases) for DTC. Our patients were treated with supraphysiological LT4 doses but were not overtly thyreotoxic, as evident from serum TT3-concentrations in the reference range. It is unclear to what extent the ThyPRO is able to assess changes in QoL in the euthyroid range/ subclinical range. Finally, we were only able to measure TFTs in serum, which might not reflect tissue concentrations as we mentioned in the introduction. In conclusion, we did not find any association between TFTs and health-related QoL nor with thyroid specific QoL and fatigue in athyreotic patients with a history of DTC. Our data do not support the hypothesis that complaints of fatigue and/or impaired well-being in hypothyroid patients on LT4 therapy are due to relatively low levels of serum T3 because of a lack of endogenous T3 secretion by the thyroid gland or to altered concentrations of T4 metabolites. The lack of association between QoL and a detailed set of TFTs in this cohort provides no evidence for the hypothesis that slight increments in LT4 and/ or T3 dose would improve fatigue or impaired well-being in hypothyroid patients.

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Supplemental table 1. Association between TFTs and other ThyPRO scales using logistic regression analysis

	Emotional susceptibility		**Social impairment**		**Impaired day life**		**Hypothyroid symptoms**		**Anxiety**	
	OR (95%CI)	*P*	OR (95%CI)	*P*	OR (95%CI)	*P*	OR (95%CI)	*P*	OR (95%CI)	*P*
TSH	0.5 (0.2-1.1)	0.08	1.01 (0.5-1.9)	0.96	0.9 (0.5-1.7)	0.72	0.89 (0.5-1.7)	0.72	1.2 (0.7-2.3)	0.51
FT4	0.99 (0.9-1.1)	0.91	1.07 (0.96-1.1)	0.42	0.99 (0.9-1.1)	0.79	1.04 (0.96-1.1)	0.35	0.99 (0.92-1.1)	0.84
TT4	0.99 (0.98-1.01)	0.27	1.0 (0.99-1.01)	0.69	0.99 (0.99-1.01)	0.75	1.01 (0.99-1.02)	0.51	0.99 (0.98-1.01)	0.15
TT3	0.56 (0.15-2.1)	0.40	1.4 (0.4-4.9)	0.58	0.67 (0.2- 2.6)	0.56	0.72 (0.2-2.9)	0.64	0.71 (0.2-2,7)	0.61
rT3	0.25 (0.01-4.9)	0.36	1.1 (0.07-17.6)	0.95	1.6 (0.09-29.9)	0.74	0.8 (0.04-15.8)	0.88	0.08 (0.004-1.7)	0.10
3,5-T2	1.14 (0.5-2.8)	0.77	1.4 (0.6-3.4)	0.43	3.1 (1.2-8.6)	0.025	1.65 (0.6-4.4)	0.31	1.2 (0.5-3.0)	0.70
T2/T3ratio	1.3 (0.3-6.4)	0.71	1.6 (0.35-7.0)	0.55	6.6 (1.1-39.5)	0.04	2.2 (0.4-11.9)	0.36	1.4 (0.3-6.6)	0.66
T4/T3ratio	1.0 (0.97-1.04)	0.76	1.0 (0.97-1.04)	0.80	0.99 (0.96-1.02)	0.55	1.02 (0.99-1.06)	0.18	0.99 (0.96-1.03)	0.78
Time since diagnosis	0.95 (0.9-1.01)	0.091	0.93 (0.9-0.99)	0.025	0.95 (0.9-1.01)	0.09	0.96 (0.9-1.02)	0.18	0.93 (0.9-0.99)	0.046
Number of drugs	1.3 (1.1-1.6)	**0.008**	1.03 (0.86-1.2)	0.77	1.08 (0.9-1.3)	0.36	1.2 (1.01-1.5)	0.048	1.1 (0.9-1.3)	0.30
Cured or not	2.5 (0.8-7.6)	0.11	1.2 (0.5-3.2)	0.68	2.4 (0.8-7.4)	0.13	2.3 (0.7-7.2)	0.16	0.6 (0.22-1.46)	0.24
BMI	1.0 (0.94-1.07)	0.91	1.03 (0.96-1.1)	0.40	0.98 (0.91- 1.05)	0.53	1.02 (0.95-1.1)	0.52	0.99 (0.92-1.07)	0.69

Supplemental table 2. Association between TFTs and MFI dimensions using multiple regression analysis

	General fatigue			Physical fatigue			Reduced motivation			Reduced activity			Mental fatigue		
	β	± SE	*P*	β	± SE	*P*	β	± SE	*P*	β	± SE	*P*	β	± SE	*P*
TSH	-0.13	± 0.13	0.30	-0.075	± 0.13	0.56	-0.12	± 0.14	0.4	-0.098	± 0.14	0.48	0.00	± 0.14	0.997
FT4	0.011	± 0.02	0.49	0.013	± 0.02	0.40	-0.001	± 0.02	0.97	0.022	± 0.02	0.20	0.002	± 0.02	0.90
TT4	-0.003	± 0.00	0.227	-0.002	± 0.00	0.53	-0.005	± 0.00	0.095	-0.001	± 0.00	0.68	-0.003	± 0.00	0.31
TT3	-0.46	± 0.26	0.075	-0.18	± 0.26	0.49	-0.31	± 0.28	0.27	-0.16	± 0.28	0.57	-0.37	± 0.27	0.17
rT3	0.066	± 0.58	0.91	0.31	± 0.58	0.59	-0.015	± 0.62	0.98	0.01	± 0.62	0.99	-0.35	± 0.60	0.55
3,5-T2	0.20	± 0.19	0.29	0.16	± 0.18	0.35	0.055	± 0.20	0.79	0.35	± 0.19	0.082	0.24	± 0.19	0.21
T2/T3 ratio	0.39	± 0.32	0.22	0.32	± 0.30	0.30	0.07	± 0.35	0.83	0.51	± 0.34	0.14	0.39	± 0.33	0.23
T4/T3 ratio	0.002	± 0.01	0.74	0.001	± 0.01	0.85	-0.006	± 0.01	0.34	0.001	± 0.01	0.90	0.00	± 0.01	0.98
Time since diagnosis	-0.014	± 0.01	0.17	-0.019	± 0.01	0.068	-0.009	± 0.01	0.43	-0.01	± 0.01	0.38	-0.02	± 0.01	0.068
Number of drugs	0.086	± 0.04	0.018	0.11	± 0.04	**0.006**	0.09	± 0.04	0.026	0.084	± 0.04	0.032	0.046	± 0.04	0.223
Cured or not	0.08	± 0.19	0.70	0.05	± 0.19	0.79	-0.19	± 0.21	0.37	-0.23	± 0.2	0.26	0.09	± 0.2	0.64
BMI	0.016	± 0.01	0.25	0.03	± 0.01	0.033	<0.001	± 0.01	0.99	0.015	± 0.01	0.29	0.004	± 0.01	0.80

PART III

Search for new biomarkers of tissue-specific thyroid state

Chapter 6

Serum microRNA profiles in athyreotic patients in different thyroid states

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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 are 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 athyreotic 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). Despite adequate restoration of biochemical euthyroidism with levothyroxine (LT4) replacement therapy, a substantial proportion of patients (~10-15%) display significant impairment of psychological well-being compared to controls of similar age and sex and show decrements in health status (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). The LT4 dose needed to normalize thyroid hormone concentrations was tissue-dependent, and supra-physiological serum T4 concentrations had to be reached in most tissues to normalize their local T3 concentrations.

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 mRNAs 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 are also 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.
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 (17), 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, Den Haag, 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 (18). 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) (19). The miRNAs with a Ct value of 40 in ≥ 75% of the samples were removed from the dataset. The non-human external control (ath-miRNA 159a) differed between the serum samples with Ct values ranging between 23.0 and 26.9 (mean Ct value 24.4; SD 0.96). In previous studies using the same protocol, variation in spike-in controls has been reported to be similar (20). To allow comparison between the individual samples, calibration and normalization was performed using global normalization according to Mestdagh *et al.*(21). 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 Ct values (±SD) of 28.3 (±0.58) and 30.5 (±0.6) respectively (22). Normalization was also performed using the mean of these normalizers.

Software and statistics

Data were extracted using ExpressionSuite v1.0.4 (Life Technologies/Applied Biosystems). Data pre-processing was performed in Microsoft Excel 2010. Statistical analysis was performed using SPSS Statistics for Windows, version 21 (IBM Corp., Armonk, NY, USA). The

Wilcoxon signed rank test was used for paired comparison of miRNA expression (ΔCt) and thyroid function tests on and off LT4 treatment. Multiple comparison adjustment was applied using the FDR approach (23).

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.
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 1. Baseline characteristics

Subject	Age	Sex	BMI (kg/m^2)	Type of tumor	Comorbidity	Number of RAI treatments	Tg-off (µg/l)	Post-therapy I^{131} 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

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 pmol/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.

Profiling of miRNAs

Out of the 384 miRNAs analyzed, almost half showed very low to undetectable levels. After removing all miRNAs with Ct values of 40 in ≥ 75% of the samples, only 195 miRNAs remained for analysis. Supplemental table 1 shows the fifty most abundant miRNAs in serum on and off LT4. 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 (Fig 1). 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 expression of any of the miRNAs analyzed between thyroid states (data not shown).

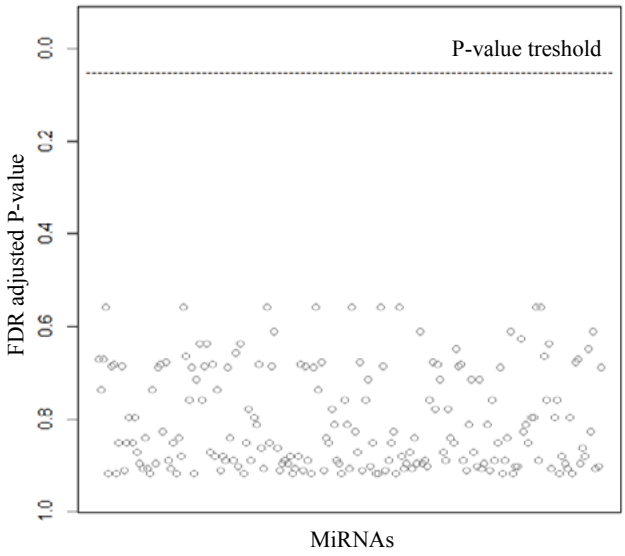

Figure 1. Scatterplot showing the false discovery rate (FDR) adjusted p-value for each miRNA separately. The dashed line represents the FDR significance threshold.

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 was mostly undetectable and showed a mean Ct value of 39.1 (±2.1) in hypothyroid state versus 38.0 (±2.3) in hyperthyroid state (not significant). MiRNA 206 was not on the card. MiRNA 133a showed a mean Ct value of 31.2 (±1.2) in hypothyroid state versus 31.3 (±1.7) in hyperthyroid state. MiRNA 133b was also mostly undetectable with a mean Ct value of 38.7 (±2.9) in hypothyroid state versus 37.9 (±3.0) in hyperthyroid state (not significant).

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 (24). 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 (25). 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 (25). Another study demonstrated that different levels of circulating miRNAs are associated with intractable Graves' disease compared with Graves' disease in remission (26).

In our study, we could not find significant changes in expression of serum miRNAs in athyreotic hypothyroid patients on and off LT4 treatment, despite previous studies showing clear differences in (precursors of) miRNAs in different tissues (13, 14, 27). 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 expression 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 (28). 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 (28). 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 (29). 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, 27). 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 (24). 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. 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 (19, 30-32). 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.

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Supplemental Table 1. Fifty most abundant miRNAs in serum off and on levothyroxine (LT4)

off LT4	mean Ct value	range	on LT4	mean Ct value	range
miRNA 223	18.4	(15.9-19.5)	miRNA 223	18.9	(17.2- 20.5)
miRNA 451	21.9	(20.8-23.7)	miRNA 451	22.3	(19.1- 24.7)
miRNA 16	22.7	(21.6-24.0)	miRNA 16	23.2	(21.6-24.4)
miRNA 126	24.5	(23.8-25.3)	miRNA 126	24.6	(23.7-25.7)
miRNA 92a	24.6	(23.9-25.2)	miRNA 92a	24.7	(23.6-26.1)
miRNA 19b	24.7	(22.9-25.6)	miRNA 19b	24.8	(23.5-26.1)
miRNA 191	24.7	(23.7-25.5)	miRNA 146a	25.0	(23.6-26.4)
miRNA 17	24.8	(24.0-25.5)	miRNA 191	25.0	(24.0-26.4)
miRNA 106a	24.8	(23.8-25.6)	miRNA 17	25.0	(24.1-25.5)
miRNA 24	24.9	(24.2-26.5)	miRNA 106a	25.1	(23.7-26.5)
miRNA 146a	25.0	(23.7-26.5)	miRNA 484	25.4	(23.9-27.0)
miRNA 20a	25.3	(24.4-26.5)	miRNA 24	25.5	(23.7-27.7)
miRNA 484	25.4	(23.2-26.5)	miRNA 20a	25.7	(24.7-26.6)
miRNA 150	25.9	(23.4-28.5)	miRNA 30c	26.3	(24.6-28.4)
miRNA 30c	26.3	(25.2-27.5)	miRNA 30b	26.6	(25.7-27.6)
miRNA 30b	26.7	(26.0-27.8)	miRNA 150	26.8	(25.1-27.9)
miRNA 26a	26.9	(26.1-27.7)	miRNA 21	26.9	(26.1-27.8)
miRNA 320	27.0	(25.5-28.5)	miRNA 222	27.1	(25.9-28.5)
miRNA 197	27.1	(25.7-28.6)	miRNA 320	27.3	(26.5-28.3)
miRNA 222	27.1	(25.6-29.3)	miRNA 26a	27.3	(26.1-32.7)
miRNA 21	27.1	(26.0-27.8)	miRNA 197	27.5	(26.0-29.6)
miRNA 3423p	27.4	(26.3-28.6)	miRNA 3423p	27.7	(26.9-28.7)
miRNA 25	27.4	(26.5-28.2)	miRNA 25	28.0	(26.3-29.4)
let7b	27.9	(25.7-30.5)	miRNA 15b	28.1	(27.5-29.4)
miRNA 26b	28.0	(26.9-29.5)	let7e	28.2	(27.0-29.6)
miRNA 15b	28.0	(26.5-30.6)	miRNA 26b	28.3	(27.0-29.6)
miRNA 93	28.2	(27.5-28.6)	miRNA 93	28.4	(27.4-29.6)
miRNA 106b	28.4	(27.5-29.5)	let7b	28.4	(26.6-32.2)
let7e	28.5	(26.9-29.7)	miRNA 221	28.4	(27.5-29.7)
miRNA 3313p	28.6	(27.4-29.6)	miRNA 3313p	28.6	(26.7-29.7)
miRNA 146b5p	28.7	(27.7-29.5)	miRNA 106b	28.7	(27.7-29.6)
miRNA 221	28.8	(28.4-28.6)	miRNA 1395p	28.7	(26.6-30.5)
miRNA 5743p	28.8	(26.8-30.5)	miRNA 145	28.9	(27.6-29.6)
miRNA 122	28.9	(26.5-30.5)	miRNA 5743p	29.0	(27.3-31.5)

Supplemental Table 1. Fifty most abundant miRNAs in serum off and on levothyroxine (LT4) *(Continued)*

off LT4	mean Ct value	range	on LT4	mean Ct value	range
miRNA 374b	29.0	(27.5-29.5)	miRNA 122	29.0	(27.1-30.6)
miRNA 145	29.0	(27.9-30.6)	miRNA 146b5p	29.1	(28.4-29.6)
miRNA 1395p	29.2	(27.5-31.0)	miRNA 148a	29.2	(27.5-30.6)
miRNA 8855p	29.3	(27.5-30.5)	miRNA 328	29.3	(28.0-31.6)
miRNA 29a	29.3	(27.8-30.7)	miRNA 660	29.3	(27.8-31.7)
miRNA 186	29.4	(28.0-30.5)	miRNA 374b	29.4	(28.5-30.6)
miRNA 20b	29.5	(28.1-30.6)	miRNA 199a3p	29.5	(28.0-30.4)
miRNA 103	29.5	(27.4-38.6)	miRNA 29a	29.6	(27.0-31.4)
miRNA 199a3p	29.6	(28.6-30.6)	miRNA 186	29.8	(28.6-30.7)
miRNA 148a	29.6	(28.6-30.8)	miRNA 20b	29.9	(28.5-30.6)
miRNA 328	29.6	(28.1-31.5)	miRNA 19a	30.0	(28.7-30.9)
miRNA 660	29.6	(28.5-30.6)	miRNA 195	30.1	(27.7-31.5)
miRNA 195	29.8	(28.5-32.4)	miRNA 103	30.1	(28.1-40)
miRNA 19a	30.0	(29.0-31.6)	miRNA 8855p	30.1	(28.9-31.6)
miRNA 335	30.2	(28.5-31.7)	miRNA 27a	30.1	(29.1-35.7)
let7g	30.2	(28.0-33.6)	let7g	30.2	(27.8-32.0)

Chapter 7

Thyroid state regulates gene expression in human whole blood

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Abstract

Context: Despite the well-recognized clinical features due to insufficient or excessive thyroid hormone (TH) levels in humans, it is largely unknown which genes are regulated by TH in human tissues.

Objective: To study the effect of TH on human gene expression profiles in whole blood, mainly consisting of TRα-expressing cells.

Methods: We performed next-generation RNA sequencing on whole blood samples from 8 athyroid patients (4 females) on and after 4 weeks off levothyroxine replacement. Gene expression changes were analyzed through paired differential expression analysis and confirmed in a validation cohort. Weighted gene co-expression network analysis (WGCNA) was applied to identify thyroid state-related networks.

Results: We detected 486 differentially expressed (DE) genes (fold-change above 1.5; multiple testing corrected *P*-value <0.05), of which 76 % were positively and 24 % were negatively regulated. Gene ontology (GO) enrichment analysis revealed that 7 biological processes were significantly overrepresented of which the process translational elongation showed the highest fold enrichment (7.3 fold, $P=1.8 \times 10^{-6}$). Comparative transcriptome analysis revealed significant overlap with DE-genes in muscle samples upon different thyroid state (2.3-fold enrichment; $P=4.1 \times 10^{-5}$). WGCNA analysis independently identified various gene clusters that correlated with thyroid state. Further GO-analysis suggested that TH regulates platelet function.

Conclusions: Changes in thyroid state regulate numerous genes in human whole blood, predominantly TRα-expressing leukocytes. In addition, TH may regulate gene expression in platelets. Whole blood samples can potentially be used as a proxy for other TRα-expressing tissues in humans.

Introduction

Thyroid hormone (TH) affects virtually all cells and tissues in the human body. The major biologically active TH is T3 and its genomic actions are mediated by binding to nuclear T3 receptors (TRs) which regulate transcription of target genes (1). Several receptor isoforms are encoded by the *THRA* and *THRB* genes of which TRα1, TRβ1 and TRβ2 are the truly T3 binding isoforms (2). The TR isoforms have a distinct expression pattern, with a predominance of TRα1 in brain, heart and bone and TRβ1 in the liver, kidney and thyroid. TRβ2 is mainly expressed in hypothalamus and pituitary and is, thus, involved in the regulation of the hypothalamus-pituitary-thyroid axis (3).

Despite the classical clinical features due to insufficient or excessive TH levels which have been recognized for over 100 years, the underlying molecular mechanisms in humans are not well understood. Knowledge of gene expression modulated by TH is largely derived from animal models or in vitro cellular studies used to explore which genes are regulated by TH (4-7). Expanding knowledge of the effects of TH on gene expression in human tissues will provide more insight in the molecular basis of TH action and may lead to a better understanding of the clinical effects of TH in humans.

Progress is limited because most human tissues are not easily accessible. However, blood can be regarded as circulating tissue and contains various cell types including erythrocytes, leukocytes and platelets (8). RNA in whole blood is largely determined by leukocytes, which mainly express the TRα isoform (9). Therefore, analysis of gene expression in whole blood may potentially be used as proxy for other TRα-expressing tissues. To study the effects of TH on gene expression in human TRα-expressing cells we performed next generation RNA sequencing (RNA-Seq) in whole blood cells from athyroid patients off and on levothyroxine (LT4) treatment.

Patients and Methods

Patients

Patients were recruited via the outpatient clinic of the Erasmus Medical Center, which is a tertiary referral center for differentiated thyroid cancer (DTC). Patients with DTC undergoing TSH-stimulated ^{131}I therapy after withdrawal of LT4 were asked to participate in the study. Patients were eligible for inclusion if they had no other malignancies or an active inflammatory disease, were not using any drugs known to influence TH metabolism, and were between 18 and 80 years old. A discovery and a validation cohort were created according to the same protocol.

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

Sample collection and serum analyses

Peripheral blood samples were obtained from all participants when overt biochemical hypothyroidism was achieved by withdrawal of LT4 substitution in thyroidectomized patients as well as when TSH suppression was achieved after restarting LT4 replacement therapy. Serum free T4 (FT4, reference range 11-25 pmol/l), total T4 (reference range 58-128 nmol/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 (reference range 0.4-4.3 mU/l) was measured by an immunometric assay (Immulite 2000 XPi, Siemens, Den Haag, the Netherlands). Whole blood samples were collected in PAXgene tubes. PAXgene tubes contain a proprietary reagent that immediately stabilizes intracellular mRNA, thus reducing mRNA degradation and inhibiting gene induction after phlebotomy. The isolated mRNA represents all blood cells, including polymorph nuclear leukocytes, mononuclear cells, platelets and red blood cells (10). RNA was isolated using PAXgene blood miRNA kits (PreAnalytiX, Hombrechtikon, Switzerland). For RNAseq analysis of the discovery cohort, ribosomal RNA and globin mRNAs were first removed from an aliquot of the RNA samples using the Globin-Zero Gold rRNA Removal Kit (Illumina, San Diego, USA) (11). Kapa Stranded RNA library was prepared (Kapa Stranded RNA kit, Kapabiosystems), followed by sequencing on a HiSeq2500 system, for single end reads, 50 bp in length (Illumina, USA).

Bioinformatic analysis

We analyzed genes with at least 5 reads in at least 6 samples. The generated sequencing reads were aligned (stranded alignment) against the GRCh38 version of the human reference genome, with RefSeq gene annotation, using Tophat2 (12). Gene counts were generated from the BAM files with HTseq (13). Cufflinks was used to compute transcript abundance estimates in fragments per kilo base of transcript per million mapped reads (FPKMs) (14). Differential expression was calculated with the bioinformatics tool DESeq 2 from Bioconductor, which uses the R statistical programming language (15). Cut off values for significantly expressed genes were a false discovery rate of 0.05 or less and a fold change of 1.5. To visualize the clustering of the samples, principal component analysis (PCA) was performed. The normalized data file was transposed and imported into OmniViz version 6.1.13 (Instem Scientific, Inc., Stone, Staffordshire, UK) for further analysis. The geometric mean of the intensity of all samples was calculated. The level of expression of each gene was determined relative to this geometric mean and ^{2}log transformed. The geometric mean of all samples was used to ascribe equal weight to gene expression levels with similar relative distances to the geometric mean. The data were deposited in NCBI/GEO reference (URL) and Accession number GSE103305.

We used functional enrichment analysis to explore the biological significance of DE-genes and of genes in WGCNA-modules (see below) using DAVID (EASE) (16). DAVID calculates

significantly overrepresentation of gene ontology (GO)-classified biological processes by comparing the number of genes in a gene list for a biological process to the number of genes for that biological process from the RNA-Seq analysis (17). Biological processes are shown which were significantly enriched after correction for multiple testing.

Quantative PCR (qPCR)

To confirm the RNA-Seq results by an independent technique, qPCR was used to measure expression of selected genes. QPCR was performed on cDNA produced from RNA before applying Globin-Zero Gold rRNA Removal Kits on the discovery cohort and on cDNA from the validation cohort. The primer sequences are presented in Supplemental Table 1. RNA levels are expressed relative to the geometric mean of the house-keeping genes GAPDH and ACTB.

Table 1. Thyroid function tests and leukocyte counts

	Off LT4	**On LT4**	***P***
TSH (mU/l)	78.0 (48.5-117.5)	0.07 (0.004-0.11)	0.012
Total T3 (nmol/l)	0.72 (0.63-0.83)	2.11 (1.94-2.28)	0.012
Total T4 (nmol/l)	17.0 (14.0-18.0)	155.0 (135.5-170.5)	0.018
FT4 (pmol/l)	1.55 (1.12-2.17)	26.2 (22.3-29.5)	0.012
Leukocytes (x10^9/l)	7.6 (5.5- 8.1)	7.4 (5.5-8.7)	0.26
Neutrophils (%)	60.0 (52.0-67.0)	62.8 (58.2-65.7)	0.50
Lymphocytes (%)	32.4 (25.1-35.9)	27.3 (23.5- 28.6)	0.13
Monocytes (%)	4.7 (4.6- 7.8)	8.1 (6.9-9.6)	0.018
Dosage LT4 (µg/kg)		2.3 (2.0-2.7)	
Time between tests (wks)		22.5 (17.2-24.0)	

Thyroid function tests and leukocyte counts off and on LT4 replacement. Data are expressed as median with IQR. Data were compared using the Wilcoxon signed rank test.

Statistical analysis

Likelihood and significance of the overlap with DE-genes in muscle samples off and on LT4 treatment previously reported were tested using the $\chi 2$-test (18). Thyroid function tests and leukocyte counts were compared off and on LT4 treatment using the Wilcoxon signed rank test. Statistical analysis was performed using SPSS Statistics for Windows, version 22 (IBM Corp., Armonk, NY, USA).

Weighted gene co-expression network analysis

We performed a weighted gene co-expression network analysis (WGCNA) to discover co-expressed TH-related networks (modules) (19, 20). In short, a weighted adjacency matrix

containing pair-wise connection strengths was constructed by using the soft-thresholding approach (β=6) on the matrix of pair-wise correlation coefficients. A connectivity measure (k) per gene was calculated by summing the connection strengths with other genes. Modules were defined as branches of a hierarchical clustering tree using a dissimilarity measure (1 - topological overlap matrix) (20, 21). Each module was subsequently assigned a color. The gene expression profiles of each module were summarized by the module eigengene (defined as the first principal component of the module expression levels). Each module eigengene was correlated to thyroid status (with age, BMI, gender, leukocytes and leukocyte differentiation as covariates) using the WGCNA R package (FDR 5%).

Results

Study population

We studied whole blood samples from 8 athyroid patients (4 females and 4 males) off and on LT4 treatment. Characteristics of the patients are shown in Supplemental Table 2. Table 1 shows the serum TH concentrations and total leukocyte counts, including leukocyte differentiation, off and on LT4 treatment. As expected, the thyroid function tests were significantly different. Neither total leukocyte counts nor leukocyte differentiation showed significant differences, except for a slight increase in monocyte number during LT4 treatment.

Validation of selected genes

To validate the RNA-Seq data from the discovery cohort, qPCR was used as an independent technique to measure the relative expression of selected DE genes. Genes were selected based on robust baseline expression, robust fold change in expression and low adjusted p-value. All upregulated and most downregulated genes showed significant differential expression using qPCR (Figure 2). We validated our findings in an independent cohort of which the baseline characteristics are shown in Supplemental Table 4. qPCR analysis of the validation cohort confirmed that all genes were similarly regulated by TH as in the discovery cohort, although this was not significant for some small nucleolar RNAs (snoRNAs) and PDE5A (Figure 2).

Standard gene expression analysis

To assess the effects of thyroid state on gene expression in whole blood cells, we performed next-generation RNA-Seq on samples drawn from patients off and on LT4 replacement therapy. At least 15 million reads were generated for each sample and approximately 95% of these reads were aligned. We first quantified both TR isoforms to confirm that TRα is the main receptor isoform expressed in whole blood samples (Figure 1A). After filtering for expressed genes, 16014 genes remained for analysis. PCA analysis showed a high degree of clustering

of patients off and on LT4 treatment. We detected 1227 differentially expressed (DE) genes (multiple testing corrected P value < 0.05). We selected 486 DE-genes with an absolute fold-change above 1.5 (Supplemental Table 3), shown as hierarchical clustering in Figure 1B. This indicates that thyroid hormone has both positive and negative effects on gene regulation (76 % upregulated; 24 % downregulated). The 47 genes that differed at least 2-fold in expression are presented in Table 2.

Table 2. Genes that differed 2-fold in expression (^{2}log ratio >1 or < -1)

^{2}Log ratio	Molecules	*P* adj.	Description
Upregulated genes			
1.824	CCNJL	2.0×10^{-24}	Cyclin J-Like
1.377	SHISA4	5.1×10^{-6}	Shisa Family Member 4
1.342	PLVAP	2.6×10^{-6}	Plasmalemma Vesicle Associated Protein
1.257	PDE5A	9.4×10^{-8}	Phosphodiesterase 5A
1.247	RIOK3	5.6×10^{-8}	RIO Kinase 3
1.247	TUBB1	7.8×10^{-8}	Tubulin, Beta 1 Class VI
1.211	ARHGEF12	1.3×10^{-7}	Rho Guanine Nucleotide Exchange Factor (GEF) 12
1.193	SH3BGRL2	5.7×10^{-7}	SH3 Domain Binding Glutamate-Rich Protein Like 2
1.179	LINC00989	7.3×10^{-6}	Long Intergenic Non-Protein Coding RNA 989
1.172	DAAM2	0.00012	Dishevelled Associated Activator Of Morphogenesis 2
1.169	CA2	6.2×10^{-6}	Carbonic Anhydrase II
1.168	CTTN	2.1×10^{-6}	Cortactin
1.165	RNF182	0.00027	Ring Finger Protein 182
1.163	ITGA2B	2.7×10^{-6}	Integrin, Alpha 2b
1.151	EIF2AK1	4.5×10^{-7}	Eukaryotic Translation Initiation Factor 2-Alpha Kinase 1
1.139	RAB27B	2.1×10^{-6}	RAB27B, Member RAS Oncogene Family
1.136	PROS1	4.8×10^{-5}	Protein S (Alpha)
1.123	CXCR2P1	1.4×10^{-5}	Chemokine (C-X-C Motif) Receptor 2 Pseudogene 1
1.114	ABCG2	7.8×10^{-5}	ATP-Binding Cassette, Sub-Family G (WHITE), Member 2
1.111	ITGB3	0.00014	Integrin, Beta 3 (Platelet Glycoprotein IIIa, Antigen CD61)
1.093	ARL4A	3.0×10^{-6}	ADP-Ribosylation Factor-Like 4A
1.082	STON2	7.8×10^{-8}	Stonin 2
1.081	CDKN1A	2.1×10^{-6}	Cyclin-Dependent Kinase Inhibitor 1A
1.075	CTNNAL1	0.0009	Catenin (Cadherin-Associated Protein), Alpha-Like 1
1.073	CTDSPL	6.7×10^{-6}	CTD (Carboxy-Terminal Domain, RNA Polymerase II, Polypeptide A) Small Phosphatase-Like
1.073	MYLK	1.6×10^{-5}	Myosin Light Chain Kinase
1.062	ITGB5	1.2×10^{-5}	Integrin, Beta 5
1.059	TUBB2A	0.0011	Tubulin, Beta 2A Class IIa

Table 2. Genes that differed 2-fold in expression (^{2}log ratio >1 or < -1) *(Continued)*

^{2}Log ratio	Molecules	*P* adj.	Description
1.041	MFAP3L	4.4×10^{-6}	Microfibrillar-Associated Protein 3-Like
1.031	MYOF	4.2×10^{-6}	Myoferlin
1.030	GNAZ	6.2×10^{-6}	Guanine Nucleotide Binding Protein (G Protein), Alpha Z Polypeptide
1.030	RNF10	4.3×10^{-6}	Ring Finger Protein 10
1.029	MKRN1	0.0008	Makorin Ring Finger Protein 1
1.026	IFIT1B	0.0018	Interferon-Induced Protein With Tetratricopeptide Repeats 1B
1.025	LTBP1	6.5×10^{-5}	Latent Transforming Growth Factor Beta Binding Protein 1
1.020	MMD	4.2×10^{-6}	Monocyte To Macrophage Differentiation-Associated
1.019	GNG11	4.8×10^{-5}	Guanine Nucleotide Binding Protein (G Protein), Gamma 11
1.011	ELOVL7	2.9×10^{-5}	ELOVL Fatty Acid Elongase 7
1.011	HORMAD1	8.4×10^{-5}	HORMA Domain Containing 1
Downregulated genes			
-1.014	SNORA80B	1.3×10^{-5}	Small Nucleolar RNA, H/ACA Box 80B
-1.064	SCARNA21	1.8×10^{-6}	Small Cajal Body-Specific RNA 21
-1.072	COL4A3	0.00017	Collagen, Type IV, Alpha 3
-1.091	SNORD10	2.4×10^{-8}	Small Nucleolar RNA, C/D Box 10
-1.140	SNORA34	6.7×10^{-6}	Small Nucleolar RNA, H/ACA Box 34
-1.167	SNORA71D	4.0×10^{-6}	Small Nucleolar RNA, H/ACA Box 71D
-1.214	RPS18P9	1.1×10^{-6}	Ribosomal Protein S18 Pseudogene 9
-1.397	SNORA49	6.3×10^{-9}	Small Nucleolar RNA, H/ACA Box 49

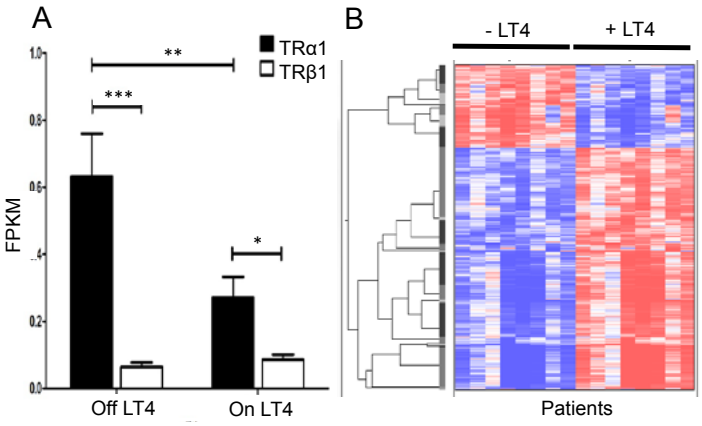

Figure 1. A. Gene expression (FPKMs) of TRα1 and TRβ1 off and on LT4 treatment. * P <0.05, **P<0.01, *** P<0.001. **B.** OmniViz Treescape shows the hierarchical clustering of DE-genes off and on LT4 treatment. Red: upregulated genes compared with the geometric mean; blue: downregulated genes compared with the geometric mean. The color intensity correlates with the degree of change.

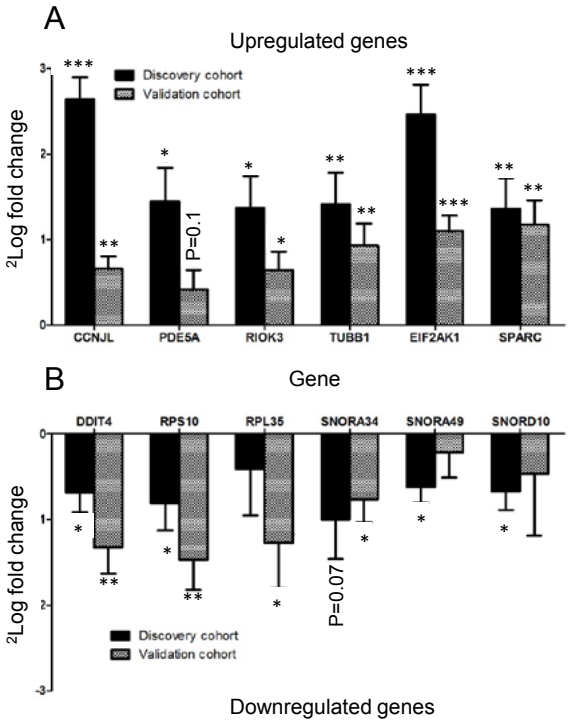

Figure 2. Verification of RNAseq results by qPCR. 6 uregulated (**A**) and 6 downregulated genes (**B**) were selected from the RNAseq results. Results are shown as 2log ratio of the fold change in gene expression off and on LT4 treatment. * P <0.05, **P<0.01, *** P<0.001.

Figure 3. GO enrichment analyses of DE-genes off and on LT4 treatment. The biological processes are shown on the Y axis.

GO analysis

Next, we analyzed whether the 486 DE-genes were associated with specific biological processes. GO enrichment analysis revealed that 7 biological processes were significantly overrepresented after correction for multiple testing (FDR 0.05) (Supplemental Table 5). Because some GO terms overlapped based on similar groups of genes, the number of biological processes was reduced to 3 (Figure 3).

Comparative transcriptome analysis

To explore to which extent gene expression in whole blood parallels gene expression in other tissues, we investigated the overlap with gene expression in skeletal muscle in different thyroid states (18). This dataset contained 607 DE-genes (fold-change > 1.5) in muscle samples off and on LT4 treatment as previously reported. Comparative transcriptome analysis of the 486 DE-genes (fold-change > 1.5) of the present study with the muscle dataset revealed 26 genes that were shared between both tissues, which is a significant 2.3-fold enrichment (P=4.1 x 10^{-5}) (Supplemental Table 6).

WGCNA

To further explore TH-dependent transcriptional patterns in the current dataset, we employed WGCNA analysis. WGCNA can reveal the underlying organization of the transcriptome based on co-expression relationships. WGCNA complements traditional DE analyses by providing a system level framework for the understanding of transcriptional profiles. This has been shown particularly helpful in transcriptome analysis of samples composed of distinct cell and tissue types (22).

Therefore, WGCNA is potentially useful to identify specific cell types responsive to different thyroid states. First, the genes showing most variability between samples (i.e. at least a 2.0-fold change in level of expression from the global mean) were selected. This resulted in 6649 genes, which were determined by their coefficient of variance, rather than any sample characteristics such as disease status. Subsequently, unsupervised hierarchical clustering led to the identification of 17 co-expression modules (Figure 4A). Modules correspond to branches and are color-coded, ranging in size from 67 genes in the light cyan module to 1737 in the turquoise module (Supplemental Table 7). Next, modules were identified that were significantly associated with clinical parameters, including age, sex, leukocytes and thyroid state. Therefore, the summary file (eigengene) for each module was correlated with the clinical parameters to select the most significant associations (Figure 4B). The strength of WGCNA analysis is well exemplified by the tan module. The unbiased approach identified 120 genes in this module, which were closely linked according to WGCNA. Subsequent inspection learned that this module mainly contained genes expressed from the Y-chromosome. Regression

analysis afterwards correctly identified (male) sex to this module (r= 0.97, p= 2.0x 10^{-10}). Using similar analysis, the blue and the midnight blue modules were significantly associated with thyroid state. The blue module correlated positively with thyroid state (r=0.65, p= 0.007) and contained numerous genes (213 of the 814 genes in this module) that were also found significantly regulated by TH in the DE analysis. The positive correlation reflects that most blue module genes were upregulated by TH.

Next, we performed a GO enrichment analysis for the genes in the blue module (Supplemental Table 5). The biological process hemostasis was significantly enriched in the blue module. Of note, many genes in the blue module appeared transcripts expressed in platelets (e.g. P2RY12, PF4). The module midnight blue was negatively correlated with thyroid state (r= -0.78, p= 4.0 x 10^{-4}) and contained mostly genes (32 of the 74 genes) that were regulated by TH as well in the DE analysis, of which 26 (75%) were downregulated. Many genes in the module midnight blue belonged to the class of snoRNAs and it contained mostly genes without a GO annotation. Thus, WGNCA analysis suggested that gene expression not only in leukocytes but also in platelets is dependent on thyroid state.

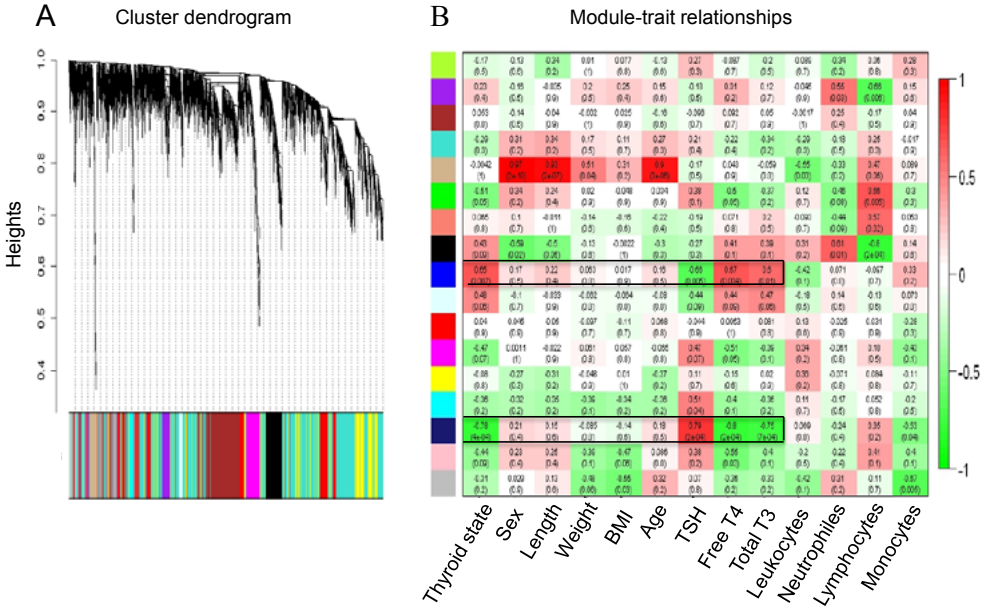

Figure 4. A. Network construction identifies distinct modules of co-expressed genes. Modules of co-expressed genes were assigned colors corresponding to the branches indicated by the horizontal bar beneath the dendrogram. **B.** Heatmap plot of the adjacencies in the eigengene network including several traits. Each row and column in the heatmap corresponds to one module eigengene (labeled by color) or trait (labeled on the x-axis). In the heatmap, green color represents low adjacency (negative correlation), while red represents high adjacency (positive correlation). The Blue and the Midnight Blue modules were significantly associated with thyroid state.

Discussion

In humans, genes that are regulated by thyroid state are largely unknown. Previously, we and others discovered genes that are dependent on thyroid state in human skeletal muscle (18, 23). *Ex vivo* studies have identified T3-responsive genes in human skin fibroblasts and adipocytes (23-25). The present study identifies numerous genes in human whole blood samples that are regulated by thyroid state. Similar to previous transcriptome analysis studies, TH largely positively regulates gene expression, although a considerable number of genes is downregulated. As RNA from leukocytes is a major determinant to total RNA in blood (after removal of globin RNA), it is likely that the detected DE-genes largely represent the effects of thyroid state on leukocytes. Indeed, lymphocytes in peripheral blood have been shown to respond to T3 (26).

To improve the yield of our genome-wide expression profiling, we performed WGCNA and compared these results with those of a standard analysis based on differential expression. While a standard analysis typically discloses lists of DE-genes, it fails to recognize the different connections between them. Indeed, WGCNA mapped many genes related to thyroid state into two large co-expression modules (modules blue and midnight blue). GO enrichment analyses of the TH-associated module blue showed predominance of the biological process hemostasis of which the involved genes were upregulated by TH. Of note, many genes were platelet specific transcripts (e.g. P2RY12, PF4). The product of *P2RY12* is a purinergic G-protein coupled receptor, which plays a crucial role in thrombus formation (27). P2RY12 inhibitors, such as clopidogrel and ticagrelor, have antithrombotic effects and are widely used in patients with acute coronary syndromes and in the secondary prevention of thrombotic events in vascular diseases (28). *PF4* codes for a chemokine (platelet factor 4) which is synthesized in megakaryocytes and stored in platelet alpha granules. When platelets are activated, PF4 is released from the alpha granules facilitating thrombosis (29). The observation that circulating PF4 levels are decreased in patients with subclinical autoimmune hypothyroidism supports our findings (30). *F13A1*, another upregulated gene by TH, encodes the coagulation factor XIIIA subunit. Coagulation factor XIII is important for stabilisation of the fibrin clot. Together, these data suggest that TH induces transcription of pro-thrombotic genes. This is in line with the observation that hyperthyroidism increases the risk of thrombosis (31, 32). Even high-normal thyroid function within the reference range is associated with stroke, independent of classical cardiovascular risk factors (33). Previously, it has been shown that the production of several coagulation factors produced in the liver is enhanced by TH (34, 35). Although we were unable to assess thrombocyte counts, other studies have demonstrated that platelet number is independent of thyroid state (36). The present study suggests that TH also positively regulates pro-thrombotic factors in platelets.

The second WGCNA-module that correlated negatively with thyroid state was mainly composed of snoRNAs which were mostly downregulated by TH. SnoRNAs are classified

into two families (box C/D snoRNAs and box H/ACA snoRNAs) and are required for posttranscriptional modifications of ribosomal RNA (rRNA)(37). As the relationship between snoRNAs and TH has not been reported before, further studies are needed to understand the consequences of this finding.

Although the effects of TH on peripheral blood cells (leukocytes, platelets) are of interest in itself (see above), we explored to which extent those findings were relevant for other tissues. As whole blood samples mainly contain TRα-expressing cells, our results likely reflect the effect of TH on gene expression via TRα. As TRα is abundantly expressed over TRβ in skeletal muscle, we sought to determine overlap between genes dependent on TH in muscle and genes identified in the present study. The significant overlap with the DE-genes from our previous study in muscle samples suggests that whole blood samples potentially can be used as a proxy for the effects of TH on other TRα-expressing tissues in humans (18). Obviously, extrapolating such findings should take into account that different factors influence TH bioactivity at the cellular level. Specifically, T3 concentrations at the pre-receptor level are importantly governed by cell-specific TH transporters and deiodinases.

Several strengths and limitations are worth mentioning to provide context to our findings. A first limitation is that the observed changes in gene expression may not necessarily reflect direct effects of TH. Gene transcription may also be indirectly dependent on TH if it modulates intermediate signaling molecules. Second, one should realize that whole blood contains many different cell types. Although WGCNA was relevant in this context, other subtle changes may have gone unrecognized. Third, DE genes in hypothyroid *versus* mild thyreotoxic state (as in this study) are not necessarily the same DE genes as in hyperthyroid *versus* mild thyreotoxic state. Finally, since the patients adhered to an iodine deficient diet at the time of hypothyroidism, direct effects of changes in iodine state on gene expression cannot be excluded. Our study has several strengths. First, the study design included paired analyses, which has the advantage to reduce confounders and variability. Second, the results were confirmed in an independent cohort, substantiating the robustness of our findings. Third, we were able to study extreme differences in thyroid state in human subjects without thyroid autoimmunity.

In conclusion, we demonstrated for the first time that thyroid state regulates numerous genes in human whole blood. Furthermore, we found that TH affects gene expression in platelets, which contributes to the understanding of thrombosis in hyperthyroidism. The overlap with previously reported DE-genes in muscle samples suggests that easily accessible whole blood samples potentially can be used as a proxy for other TRα-expressing tissues in humans. Future studies should explore if specific transcripts in whole blood can be useful biomarkers for tissue specific thyroid state.

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Supplemental Tables 3, 5 and 7 are available upon request.

Supplemental Table 1.

Gene	Sense primer (5'-3')	Antisense primer (5'-3')
ACTB	GTA CAG GTC TTT GCG GAT GT	CAC TCT TCC AGC CTT CCT TC
GAPDH	GAG TCC TTC CAC GAT ACC AAA G	GGT GTG AAC CAT GAG AAG TAT GA
RPS10	CTG GAT ACC CTC ATT GGT AAG G	GCC ATG CAG TCT CTC AAG T
DDIT4	CAG GGC GTT TGC TGA TGA AC	GGT TCG CAC ACC CAT TCA AG
EIF2AK1	CTT CTG CTC GCT CCA TTT CT	TAC GTC CAG AGT GGG TAC TT
TUBB1	AAT GCT GTC CAT CGT CCC AG	GCT GGA GAG AAT CAG CGT GT
PDE5A	GCT AAC GAC TGT AGC TCT CTT G	CCA GAA CAC GCA GAT GTA TGA
SNORD10	CTT CCC AGG GCT GTT GTC TG	CTG CTC TCA GAG TAC AAA GAC TGA
RIOK3	CTT CAT TTG ATG CGG CAG TTA T	CGA TCA ACC AGA CCT TTC CA
SNORA34	TGA CTG AAG ACC AGC AGT TGT	AAC AGC CAA CAG ACC ACA GG
RPL35	TTG CCC GTG TTC TCA CAG TT	GTC TTC TTA GGC CGC AGG TC
SNORA49	GCC TGT AAA GTG CTC TGG CA	GCC CCA AAA GAA AAC AGC GT
CCNJL	CAG GCA CCA GAG GGC AC	GGC TCA TCC ATC ATC GCG TA
SPARC	CCGCTTTTTCGAGACCTGTG	ATCCGGTACTGTGGAAGGAGT

Supplemental Table 2. Characteristics of the patients in the discovery cohort.

Subject	Sex	Ethnicity	Age (yrs)	BMI	Co-morbidity	Number of RAI	Tg Off LT4	Tg On LT4
1	female	Iranian	21	22.3	Hypoparathyroidism	2	<0.9*	<0.9*
2	male	Dutch	66	23.6	DM type 1	1	<0.9*	<0.9*
3	male	Turkish	61	28.1	No	1	3.9	<0.9
4	female	Dutch	48	33.4	No	1	1.9	<0.9
5	male	Dutch	55	53.8	Chronic renal insuff DM type 2	1	<0.9	<0.9
6	male	Dutch	60	25.0	Hypoparathyroidism	1	73.0	1.10
7	female	Moroccan	26	33.1	No	1	2.7*	<0.9
8	female	Turkish	37	24.8	Hypoparathyroidism	2	<0.9*	<0.9

* anti-Tg positive

Supplemental table 4. Characteristics and thyroid function tests of study participants in the validation cohort. Data are presented as median (IQR).

	Off LT4	On LT4	*P*
TSH (mU/l)	64.6 (57.8-142.5)	0.016 (0.008-0.17)	0.008
Total T3 (nmol/l)	0.68 (0.54-0.81)	2.02 (1.74-2.17)	0.008
FT4 (pmol/l)	1.7 (1.2-2.0)	27.3 (23.5-30.2)	0.008
Age (years)		32.0 (26.5-53.0)	
BMI (kg/m2)		28.4 (22.6-30.7)	
Time between tests(wks)		18.7 (16.0-33.2)	
Dose LT4 (µg/kg)		2.25 (1.97-2.45)	
Sex		5 males, 4 females	

Supplemental Table 6 Overlapping genes between differentially expressed genes (1.5 fold change; FDR 0.05) in skeletal muscle and whole blood samples on *versus* off LT4 treatment

^{2}Log Ratio muscle	^{2}Log Ratio whole blood	Genes	Description	Location	Type
0.79	0.72	ACSL1	acyl-CoA synthetase long-chain family member 1	Cytoplasm	enzyme
0.62	0.67	BSG	basigin (Ok blood group)	Plasma Membrane	other
0.67	0.78	C1QA	complement component 1, q subcomponent, A chain	Extracellular Space	other
1.04	-0.70	COL4A4	collagen, type IV, alpha 4	Extracellular Space	other
0.92	0.69	DAB2	disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila)	Cytoplasm	other
1.91	0.69	DNAJA4	DnaJ (Hsp40) homolog, subfamily A, member 4	Nucleus	other
-0.59	-0.65	GAS5	growth arrest-specific 5	Unknown	other
-0.75	-0.69	GPX7	glutathione peroxidase 7	Cytoplasm	enzyme
1.12	0.72	MAP2K3	mitogen-activated protein kinase kinase 3	Cytoplasm	kinase
-0.61	0.60	NUDT4	nudix (nucleoside diphosphate linked moiety X)-type motif 4	Cytoplasm	phospha-tase
-0.62	-0.64	PER1	period homolog 1 (Drosophila)	Nucleus	other
-0.68	0.80	RAPH1	Ras association (RalGDS/AF-6) and pleckstrin homology domains 1	Plasma Membrane	other
0.71	-0.60	SBK1	SH3-binding domain kinase 1	Cytoplasm	kinase
0.60	0.59	SCYL2	SCY1-like 2 (S. cerevisiae)	Cytoplasm	other
0.97	0.82	SEC14L5	SEC14-like 5 (S. cerevisiae)	Cytoplasm	other
0.87	0.61	SERPINB1	serpin peptidase inhibitor, clade B (ovalbumin), member 1	Cytoplasm	other

Supplemental Table 6 Overlapping genes between differentially expressed genes (1.5 fold change; FDR 0.05) in skeletal muscle and whole blood samples on *versus* off LT4 treatment *(Continued)*

^{2}Log Ratio muscle	^{2}Log Ratio whole blood	Genes	Description	Location	Type
0.67	0.89	SLC14A1	solute carrier family 14 (urea transporter), member 1 (Kidd blood group)	Plasma Membrane	trans-porter
0.80	0.99	SPARC	secreted protein, acidic, cysteine-rich (osteonectin)	Extracellular Space	other
0.95	1.08	STON2	stonin 2	Cytoplasm	other
1.01	0.61	TNFSF10	tumor necrosis factor (ligand) superfamily, member 10	Extracellular Space	cytokine
-0.79	0.62	ZAK	sterile alpha motif and leucine zipper containing kinase AZK	Cytoplasm	kinase
0.73	0.82	FSTL1	Follistatin-Like 1	Extracellular Space	myokine
0.59	0.85	PCSK6	Proprotein Convertase Subtilisin/ Kexin Type 6	Cytoplasm	enzyme
-0.70	1.04	MFAP3L	microfibrillar-associated protein 3-like	Plasma Membrane	kinase
1.54	0.61	MAFB	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian)	Nucleus	tran-scription factor
0.61	0.78	SLC22A4	solute carrier family 22 (organic cation/ergothioneine transporter), member 4	Plasma Membrane	trans-porter

GENERAL DISCUSSION

Chapter 8

General discussion

Hypothyroidism is one of the most common endocrine disorders with a prevalence of between 0.2% and 5.3% in Europe, depending on the definition used (1). Hypothyroid symptoms include cold intolerance, constipation, bradycardia and weight gain. In contrast, patients with hyperthyroidism complain of sweating, palpitations and weight loss. These clinical effects of alterations in thyroid state arise from changes in thyroid hormone action at the cellular level. The standard treatment for primary hypothyroidism is substitution with synthetic levothyroxine (LT4). The peripheral conversion of LT4 to the biologically active thyroid hormone T3 makes it possible to achieve normal circulating T3 concentrations. In contrast, in euthyroid patients about 20% of circulating serum T3 is directly derived from thyroidal secretion. In a subset (~10-15%) of patients, symptoms of hypothyroidism persist despite serum thyroid hormone concentrations within the laboratory reference range (2, 3). The reasons and solutions for these persisting symptoms are still a matter of debate, but these persisting symptoms can be very frustrating for both patients and doctors. Therefore, it is crucial that the pathophysiology underlying persisting complaints in patients with adequately treated hypothyroidism should be elucidated in order to improve treatment of hypothyroidism. As a first step, the physiological and molecular effects of well-known clinical features due to changes in thyroid state should be understood.

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 (<0.1 mIU/L). Since large changes in thyroid hormone levels are observed, this patient group offers the unique opportunity to observe the effect of extreme differences in thyroid state on physiological parameters within the same subject.

In the first part (**chapter 2 and 3**) of this thesis the effects of thyroid hormone on different organs was assessed to get a better understanding of physiological effects of thyroid hormone. In the second part (**chapter 4 and 5**), the association of autoimmune thyroid disease with mood disorders was studied as well as the association of thyroid function tests with quality of life (QoL). In the third part (**chapter 6 and 7**), we searched for new biomarkers of thyroid state.

Physiological effects of thyroid hormone

To get a better understanding of the clinical effects of thyroid hormone, the consequences of hypothyroidism in several target tissues was studied. In **chapter 2**, the effect of thyroid hormone on renal concentrating ability and renal function was studied in patients with DTC off and on LT4 treatment. 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 a well-known consequence of hypothyroidism (4-

6). However, in contrast with previously published studies in rats (7) and humans with autoimmune hypothyroidism/ myxoedema (8, 9), we could not detect an impairment of urinary concentrating ability during severe short-term hypothyroidism in our patients. It has to be mentioned that our LT4 treated patients received a high dosage of LT4 in order to reach TSH-suppression and that the urine osmolality was somewhat low in both thyroid states (< 1000 mOsmol/kg). One could speculate that there could have been a concentrating defect in both thyroid states precluding a statistical significant difference in urine osmolality. Future studies should include an euthyroid control group to rule out this possibility.

In **chapter 3**, the effect of thyroid hormone on the microcirculation and on the macrocirculation was studied in DTC patients off and on LT4 treatment. Normal thyroid hormone levels are required to maintain normal cardiovascular function and important changes in cardiac structure and function have been reported in patients with overt and subclinical hypothyroidism (10). Although effects of thyroid hormone on the macrocirculation have been studied extensively, very little is known of the effects of TH on the microcirculation in humans (11, 12). In hamster cheek pouch, topical application of T3 induced dose-dependent dilation of arterioles within 2 minutes of administration (13). This T3-induced dilation was countered by the inhibition of nitric oxide (NO) synthase with NG-nitro-L-arginine methyl-ester (L-NAME), suggesting that the direct effects of T3 on the in vivo microcirculation are mediated by NO through non-genomic mechanisms of action. After stimulation with nitric oxide, soluble guanylyl cyclase generates cyclic guanosine monophosphate (cGMP), which induces vasodilation (14). Interestingly, this is in line with findings from **chapter 7**. In **chapter 7,** we performed next generation RNA sequencing on whole blood samples from another cohort of DTC patients in different thyroid states. The genes *GUCY1A3* and *GUCY1B3*, which encode for the alpha and beta subunits of the enzyme soluble guanylyl cyclase respectively, were significantly upregulated by thyroid hormone. In addition, we found that the biological process vasodilation was 6.7 fold enriched by thyroid hormone (P value 0.02, not significant after correction for multiple testing). The genes in this biological process included *PDE5A,* which was significantly upregulated by thyroid hormone and encodes for a cGMP-binding phosphodiesterase. It is involved in the regulation of intracellular concentrations of cyclic nucleotides and is important for smooth muscle relaxation in the cardiovascular system (15). Although these genes were expressed in whole blood samples in our study, the findings might indicate that the same genomic effects of thyroid hormone occur in endothelial cells of blood vessels, although this needs to be confirmed in future studies.

Given all these indications that thyroid hormone affects the vasculature, we hypothesized that differences in thyroid states would also affect the microcirculation. However, we were not able to demonstrate alterations in the sublingual microcirculation, assessed with a video microscope, in patients on and off LT4 treatment, although several well-known effects on heart rate, pulse pressure, systemic vascular resistance, and peripheral perfusion were

observed. The observed alterations in peripheral perfusion are likely to be mediated by effects of thyroid hormone on thermoregulation via the skin. In hypothyroidism, the reduced central thermogenesis is partially compensated by cutaneous vasoconstriction, which might have accounted for the observed decrease in peripheral perfusion in our patients (16, 17). The sublingual (central) microcirculation is not involved in thermoregulation, which may explain why no significant changes in the sublingual microcirculation were observed.

Cognitive effects of thyroid hormone

As was mentioned above, despite adequate restoration of biochemical euthyroidism with LT4 replacement therapy, a substantial number of patients (~10-15 %) display significant impairment of psychological well-being and show decrements in health status (2, 3, 18, 19). It has been speculated that the presence of associated autoimmune diseases or thyroid autoimmunity per se may also account for these persisting symptoms and for the often reported depressive symptoms (20, 21). It has been shown that subjects with thyroid autoimmunity, who are still euthyroid, have a higher risk to develop mood disorders (22). This finding, in combination with several other findings that were mentioned in the introduction of this thesis, suggests that there might be a shared immune pathogenesis for both autoimmune thyroid disease (AITD) and mood disorders. To explore the link between AITD and mood disorders, we determined 5 growth and differentiation factors that have repeatedly been shown to be abnormally expressed in the circulation of mood disorder patients and that are capable of influencing immune and/or neuronal cell growth in **chapter 4** (23-25). These factors were studied in the serum of subjects from the Amsterdam AITD cohort (euthyroid females with at least one first or second degree relative with AITD) and were combined with levels of previously assessed immune factors (26). A distinct pattern of four inter correlating immune factors preceded TPO-Ab seroconversion in the next 5 years. This opens avenues for developing assays for the detection of individuals at risk for thyroid autoimmunity. However, this would only be helpful when one would be able to stop the autoimmune process and when thyroid dysfunction could be prevented.

Brain-derived neurotrophic factor (BDNF) levels were significantly lower in sera of the subjects from the Amsterdam AITD cohort than in healthy controls, while Insulin-like Growth Factor-Binding Protein 2 (IGFBP-2) and Epidermal Growth Factor (EGF) levels were significantly higher in the subjects. It has been demonstrated that BDNF levels are decreased in patients with a major depressive disorder and are also associated with clinical changes in depression (27). Unfortunately, we were not able to assess the association between the levels of these growth and differentiation factors and the mood state of the relatives. It would be very interesting to study the growth and differentiation factors in AITD patients with persisting symptoms, and to assess the association with these symptoms.

However, after thyroidectomy, cured patients with DTC on LT4 replacement therapy have also been shown to have an impaired QoL although they are not affected by an autoimmune disease (28, 29). This suggests that autoimmunity cannot be the only explanation for the persisting symptoms in treated hypothyroid patients. Another explanation might be the inadequacy of LT4 treatment to restore physiological T4 and T3 concentrations in serum and tissues (30-32). Studies in hypothyroid rats have demonstrated that LT4 monotherapy is not able to normalize T4 and T3 concentrations in all tissues (33). Also, it remains unknown whether other endogenous thyroid hormone metabolites with T3-agonistic or antagonistic activities, like 3,5-T2, might play a role in the persisting symptoms and decreased QoL among hypothyroid patients. Therefore, in **chapter 5**, we performed a cross-sectional study in (mostly) cured athyreotic DTC patients on LT4 monotherapy to study the association between extensive thyroid function tests and QoL. Health-related QoL (RAND-36), thyroid specific QoL (ThyPRO) and fatigue (Multidimensional Fatigue Inventory) were assessed. We confirmed that health-related QoL was impaired in our patients compared with a Dutch reference group (34). Multiple linear regression analyses did not show any association between QoL and the different TFTs, including T4/T3 and 3,5-T2/T3 ratios reflecting peripheral metabolism. Therefore, our data do not support the hypothesis that complaints of fatigue and/or impaired well-being in hypothyroid patients on LT4 therapy are due to relatively low levels of serum T3 because of a lack of endogenous T3 secretion by the thyroid gland or to altered concentrations of T4 metabolites. Furthermore, our data do not support therapy with liothyronine (T3) in patients with persisting complaints since we could not find an association with T3 concentrations and T4/T3 ratio and QoL. Since most of our patients were treated with supraphysiological LT4 doses, we cannot rule out that there might be a relationship between T3 levels and QoL in patients treated with physiological LT4 doses, although subgroup analysis in our study did not demonstrate this. However, health-related QoL and years since diagnosis showed some relation and since similar results have been reported for duration of cure in other cohorts of DTC patients (28), QoL seems to improve when time since diagnosis increases. This message is important to convey to patients, since it will give them some hope for improvement of their complaints in the future.

Search for new biomarkers of tissue-specific thyroid state

Serum TSH concentrations reflect the pituitary feedback to serum levels of thyroid hormone and is used in clinical practice to monitor LT4 treatment. Several studies have however shown that serum TSH may not always be an appropriate marker in hypothyroid patients under LT4 therapy and that normal serum TSH levels in patients receiving levothyroxine strictly only reflect pituitary euthyroidism (35-37). For this reason, novel markers representing tissue thyroid state are needed. In **Chapter 6,** we studied if microRNAs in serum are regulated by thyroid state in a cohort of DTC patients. Previously, we reported on gene expression profiles

in skeletal muscle of hypothyroid patients off and on LT4 therapy and found a large down-regulation in expression of some pri-miRNAs (38). It has been shown that miRNAs are present in the circulation (39) and have been associated with a variety of diseases (40). We therefore hypothesized that miRNA profiles in serum could also be influenced by thyroid state, and that miRNAs derived from different tissues potentially reflect tissue-specific differences in thyroid states. A magnetic bead capture system was used to isolate 384 miRNAs from serum. Subsequently, the TaqMan Array Card 3.0 platform was used for profiling. Unfortunately, a paired analysis did not demonstrate any significantly different expressed miRNA off and on LT4 treatment. In **chapter 6,** several possible explanations for these findings are provided. Our method might have been not sensitive enough to assess small differences in expression. However, only miRNAs that are extremely up- or downregulated will be suitable as clinical biomarkers and we certainly did not find such a miRNA in our selection of miRNAs. Of course, we cannot rule out that other miRNAs, which were not on the array card, are regulated by thyroid hormone in serum.

As was mentioned in the introduction, genomic actions of thyroid hormone are mediated by binding to nuclear T3 receptors, which regulate transcription of target genes (41). Thyroid hormone responsive genes have also been identified in multiple human tissues, including for example liver, muscle, skin fibroblasts and adipocytes (38) (42, 43). However, to study the effects of thyroid hormone on gene expression in human tissues, easy accessible tissues would ideally be available, like blood samples. Therefore, we performed next generation RNA sequencing in whole blood samples from DTC patients off and on LT4 treatment (**chapter 7**). We detected 1227 differentially expressed (DE) genes of which 486 genes had a fold-change above 1.5. As we confirmed that the whole blood samples contained mainly TRα expressing cells our results presumably reflect the effect of thyroid hormone on gene expression via the TRα receptor. A significant overlap with the previously reported DE-genes in muscle samples (also a TRα-tissue) was found, which suggests indeed that easily accessible whole blood samples potentially can be used as a proxy for other tissues in humans.

Weighted gene co-expression network analysis (WGCNA) was applied to identify thyroid state-related networks (modules). Two different modules that correlated significantly with thyroid state were identified. Both modules contained a lot of DE-genes from the standard differential expression analysis. The midnight blue module, which correlated negatively with thyroid state, contained numerous small nucleolar RNAs (snoRNAs). As the relationship between snoRNAs and thyroid hormone has not been reported before, further studies are needed to understand the consequences of this finding. Gene ontology analysis of the genes in the blue module, which correlated positively with thyroid hormone, revealed a 4 fold enrichment of the biological process hemostasis. This process was also significantly enriched in the 486 DE-genes from the standard DE-analysis. It is well known that hyperthyroidism increases the risk of thrombosis and that several coagulation factors are upregulated by

thyroid hormone (44-46). Our study now demonstrates an effect of thyroid hormone on gene transcription in platelets. Several genes (e.g. *P2RY12*, *PF4*) involved in coagulation and hemostasis were upregulated by thyroid hormone. *P2RY12* is a G-protein coupled receptor, which plays a crucial role in thrombus formation (47). P2RY12 inhibitors, such as Clopidogrel, have antithrombotic effects. It would be interesting to study if the amount of expression of P2RY12 predicts the risk of developing thrombosis in patients with hyperthyroidism and if patients with severe and difficult to control hyperthyroidism should temporarily be treated with P2RY12 inhibitors.

Conclusions and future directions

The studies presented in this thesis provide more insights into the physiological effects of thyroid hormone on different tissues, although we did not unravel the mechanism behind persistent complaints in patients with thyroid dysfunction yet. Future studies are warranted to study the effects of other thyroid hormone metabolites on tissues and on QoL. Some products of peripheral thyroid hormone metabolism, like thyronamines (TAMs), were previously considered as inactive breakdown products. However, several reports have recently shown that they may have relevant biological effects as well (48, 49). The structure of the thyronamines differs from T4 and deiodinated thyroid hormone derivatives by the absence of a carboxylate group on the alanine side chain. 3-Iodothyronamine (T1AM) and its metabolite T0AM have been detected in sera of humans (48, 50, 51). Pharmacological administration of 3-T_1AM has been shown to result in dose-dependent reversible effects on body temperature, cardiac function, energy metabolism, and neurological functions (52-55). However, the physiological relevance of these actions is still unclear. Furthermore, since large differences of 3-T_1AM concentrations in human serum have been reported measured by liquid chromatography-mass spectrometry (LC-MS/MS) versus a monoclonal antibody-based immunoassay, one should be very careful with interpreting the results of these studies. In **chapter 5**, we studied the association between QoL and thyroid function tests cross-sectional in athyreotic patients. A prospective study in patients, before and after thyroidectomy, assessing the association between QoL and a more extensive thyroid hormone profile (including TAMs) is needed. It is also important to include patients with benign thyroid disease (e.g. goiter, Graves' disease) to exclude an effect of having a malignancy on QoL.

In addition, the observed aberrancies in hematopoietic/ neuronal growth and differentiation factors reported in **chapter 4** need to be studied in patients who have already developed AITD. It would be very interesting to measure the growth and differentiation factors in AITD patients on LT4 substitution therapy, with and without persisting symptoms, and to assess the association between the growth and differentiation factors with these symptoms.

Although, we did not discover a new biomarker for tissue-specific thyroid state yet, we detected numerous genes which are regulated by thyroid hormone in human whole blood. Our results

demonstrate the power of untargeted approaches to detect novel pathways of thyroid hormone action. Since we found several genes in whole blood samples that were also regulated by thyroid hormone in human skeletal muscle samples, future studies should explore if specific transcripts in whole blood can be useful biomarkers for certain thyroid-related endpoints.

Finally, other technologies have emerged to detect new biomarkers. Recently, a plasma proteome and metabolome characterization (OMICS approach) of an experimental human thyrotoxicosis model detected 65 metabolites and 63 proteins which were associated with serum FT4 (56). A subset of 15 molecules allowed a good prediction of thyroid hormone function (AUC=0.86) without prior information on TSH or FT4 levels. It would be interesting to use these technologies in hypothyroid conditions as well.

This thesis introduces several important approaches in understanding the biological relevance of thyroid hormone replacement therapy and, hopefully, will stimulate further research to unravel the mechanism behind persistent complaints in patients with thyroid dysfunction.

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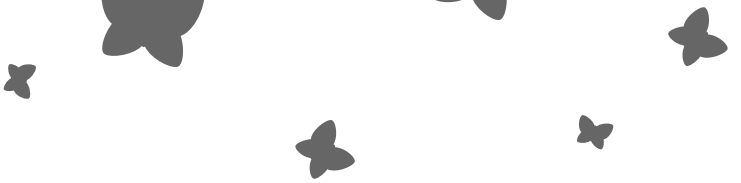

Chapter 9

Summary

Samenvatting

Summary

Thyroid hormone affects virtually all cells and tissues in the body. The thyroid produces mainly T4 (thyroxine) which is an inactive form of thyroid hormone. T4 can be converted by deiodinases into the biologically active thyroid hormone, T3 (triiodothyronine). Genomic actions of thyroid hormone are mediated by binding of T3 to nuclear T3 receptors, which regulates transcription of target genes. Clinical effects of alterations in thyroid state arise from changes in thyroid hormone at the cellular level. Hypothyroid symptoms include cold intolerance, constipation, bradycardia and weight gain. In contrast, patients with hyperthyroidism complain of sweating, palpitations and weight loss. The standard treatment of hypothyroidism is substitution with synthetic levothyroxine (LT4). However, in a subset (~10-15%) of patients, symptoms of hypothyroidism persist despite serum thyroid hormone concentrations within the laboratory reference range. It is unclear what mechanisms underlie such symptoms. In this thesis several studies were dedicated to explore the physiological and molecular processes that may explain well-known clinical observations.

The background of thyroid hormone synthesis, of thyroid hormone action and the regulation of serum thyroid hormone levels by the hypothalamus-pituitary-thyroid (HPT) axis are introduced in **chapter 1**. In addition, the outline of this thesis, which consists of three major parts, is presented in this first chapter.

Part I: Physiological effects of thyroid hormone

The first part of the thesis includes 2 studies on the physiological effects of thyroid hormone on target tissues/organs. In **chapter 2**, the effect of thyroid hormone on renal concentrating ability was studied in 9 patients off and on levothyroxine (LT4) treatment after a water and food deprivation test of 14 hours. We used serum and urine samples of patients with differentiated thyroid cancer (DTC) because they are subject to severe hypothyroidism before radioactive iodine therapy to stimulate radioactive iodine uptake by malignant tissues, whereas they have relatively high thyroid hormone concentrations afterwards when receiving substitution therapy with LT4 in order to reach TSH suppression. Our study confirmed the well-known effects of thyroid hormone on serum creatinine concentrations but could not demonstrate a significant difference in urine osmolality nor in serum osmolality on and off LT4 treatment.

In **chapter 3** a fully integrated assessment of macrocirculation and microcirculation was performed in 10 DTC patients off and on LT4 treatment. A handheld video microscope was used to visualize the sublingual microcirculation. Peripheral perfusion was assessed by several non-invasive measurements, such as Tskin-diff and the peripheral perfusion index (PFI). In addition, transthoracic echocardiography was performed to assess alterations in cardiac function. Several well-known effects of thyroid hormone on end diastolic volume and stroke volume and on pulse pressure and heart rate were confirmed. The increase in Tskin-diff

during hypothyroidism and the decreases in PFI indicated a decrease in peripheral perfusion. However, we could not demonstrate significant alterations in the microcirculation in different thyroid states.

Part II: Cognitive effects of thyroid hormone

In the second part of this thesis, the link between autoimmune thyroid disease (AITD) and mood disorders was explored (**chapter 4**) and the association between thyroid hormones and quality of life (QoL) was studied (**chapter 5**). To study the link between AITD and mood disorders several growth and differentiation factors were determined that have repeatedly been shown to be abnormally expressed in the circulation of mood disorder patients and that are capable of influencing immune and/or neuronal cell growth (**chapter 4**). We studied 64 TPO-Ab-negative females with at least 1 first- or second-degree relative with AITD, 32 of whom did and 32 who did not seroconvert to TPO-Ab positivity in 5-year follow-up. Subjects were compared with 32 healthy controls. We found that relatives of AITD patients show aberrant serum levels of 4 hematopoietic/neuronal growth factors similar to the aberrancies found in mood disorder patients. This might suggest that shared growth and differentiation defects in both the hematopoietic and neuronal system may underlie thyroid autoimmunity and mood disorders.

In **chapter 5**, the association between extensive thyroid function tests and QoL was assessed in 143 athyreotic DTC patients on LT4 replacement therapy. Health-related QoL (RAND-36), thyroid specific QoL (ThyPRO) and fatigue (Multidimensional Fatigue Inventory) were assessed, and their association with thyroid function tests was studied. Health-related QoL was impaired in our patients compared with a Dutch reference group. However, we did not find any association between TFTs, including 3,5-T2 concentrations and T4/T3 ratios, and QoL within our study group. Our data do not provide evidence that a slight increase in dose improves fatigue or well-being in hypothyroid patients on LT4 therapy.

Part III: Search for new biomarkers of tissue-specific thyroid state

In the third part of this thesis (**chapters 6 and 7**), we searched for new biomarkers of thyroid state. In **Chapter 6**, we studied for the first time if microRNAs in serum are regulated by thyroid state in 13 DTC patients off and on LT4. The expression of 384 microRNAs was assessed with a TaqMan MicroRNA Array. The generated selected serum miRNA profile did not reveal new biomarkers for thyroid state.

In **chapter 7**, next generation RNA sequencing was performed in whole blood samples in a cohort of 8 other DTC patients on and off LT4 treatment. We demonstrated that numerous genes are regulated in different thyroid states. As we confirmed that the whole blood samples

contained mainly TRα expressing cells our results presumably reflect the effect of thyroid hormone on gene expression via the TRα receptor. A significant overlap with previously reported differentially expressed genes in muscle samples was observed, which suggests that easily accessible whole blood samples potentially can be used as a proxy for other tissues in humans. In addition, it was found that thyroid hormone affects gene expression in platelets, which might explain the reported increased risk of thrombosis in patients with hyperthyroidism.

In **chapter 8**, the results which are presented in this thesis, are discussed and recommendations for future studies are suggested.

Samenvatting

De schildklier is een klein vlindervormig orgaan dat zich voor het schildklierkraakbeen in de nek bevindt. De schildklier produceert hoofdzakelijk T4 (thyroxine), hetgeen een inactieve vorm van schildklierhormoon is. T4 kan door speciale enzymen (dejodases) worden omgezet in het biologisch actieve schildklierhormoon T3 (trijodothyronine). Het belangrijkste werkingsmechanisme van schildklierhormoon verloopt via binding van T3 aan de T3-receptoren in de celkern. Wanneer T3 bindt aan zijn receptor, kunnen vervolgens schildklierhormoongevoelige genen worden afgelezen.
Schildklierhormoon beïnvloedt vrijwel alle cellen en weefsels in het lichaam. Hypothyreoïdie, te weinig schildklierhormoon, kan leiden tot koude-intolerantie, constipatie, moeheid en gewichtstoename. Patiënten met te veel schildklierhormoon, dat wil zeggen hyperthyreoïdie, hebben juist last van overmatig transpireren, hartkloppingen en gewichtsverlies. Patiënten met hypothyreoïdie worden behandeld met het synthetisch schildklierhormoon levothyroxine. Helaas blijft een deel van deze patiënten, ondanks dat hun schildklierhormoonwaarden in het bloed weer binnen de normaalwaarden zijn, klachten houden. De oorzaak hiervan wordt nog niet goed begrepen. In dit proefschrift proberen we dit probleem beter te begrijpen. Daarvoor is het van belang om de onderliggende mechanismen van de werking van schildklierhormoon op diverse weefsels beter te begrijpen. **Hoofdstuk 1** geeft algemene informatie over de schildklierhormoonfysiologie en over de inhoud van dit proefschrift. Vervolgens is het proefschrift in drie delen opgedeeld.

Deel I: Fysiologische effecten van schildklierhormoon

In dit deel van het proefschrift worden fysiologische effecten van schildklierhormoon op doelorganen beschreven. In **hoofdstuk 2** wordt het effect van schildklierhormoon op het concentrerend vermogen van de nier en nierfunctie onderzocht. We hebben dit in 9 patiënten met goed gedifferentieerd schildkliercarcinoom (schildklierkanker) onderzocht. Deze patiënten hadden vanwege een behandeling met radioactief jodium eerst verlaagde en daarna normale schildklierhormoonconcentraties in hun bloed. Voorafgaand aan de behandeling met radioactief jodium dienden deze patiënten te stoppen met het innemen van hun schildklierhormoontabletten. Hierdoor steeg de concentratie van het schildklier stimulerend hormoon (TSH), dat de opname van radioactief jodium in de tumorcellen bevordert. Alle patiënten hebben op twee momenten (tijdens hypothyreoïdie en tijdens behandeling met schildklierhormoon) 14 uur lang gevast, waarna er bloed werd afgenomen en urine moest worden ingeleverd. Nader onderzoek hiervan bevestigde de reeds bekende effecten van schildklierhormoon op serum creatinine concentraties en op klaring. We vonden echter geen effect op het concentrerend vermogen van de nier.

In **hoofdstuk 3** wordt het effect van schildklierhormoon op de microcirculatie beschreven in 10 patiënten met goed gedifferentieerd schildkliercarcinoom. Hiervoor werd een speciale videomicroscoop gebruikt, waarmee de werking van de vaatjes (de microcirculatie) onder de tong in beeld gebracht kon worden. Daarnaast werden nog diverse niet-invasieve andere technieken gebruikt om de perifere doorbloeding in kaart te brengen. Tevens werd de werking van het hart bestudeerd met echobeelden. De reeds bekende effecten van schildklierhormoon op eind-diastolisch volume, slagvolume en polsfrequentie werden bevestigd. Verder zagen we tijdens hypothyreoïdie een toename in temperatuurverschil tussen vinger en onderarm en nam de perifere perfusie-index af. Dit wijst allemaal op een verminderde perifere perfusie en koelere vingers. We konden echter geen significante veranderingen in de microcirculatie vinden tussen de verschillende schildklierhormooncondities.

Deel II: Cognitieve effecten van schildklierhormoon

In het tweede deel van dit proefschrift wordt in **hoofdstuk 4** de link tussen auto-immuun schildklierziekten en stemmingsstoornissen onderzocht en in **hoofdstuk 5** de associatie tussen schildklierhormoon en kwaliteit van leven.

In **hoofdstuk 4** worden diverse groei en differentiatie factoren beschreven, waarvan is aangetoond dat ze afwijkend tot expressie komen in het serum van patiënten met een stemmingsstoornis en waarvan bekend is dat ze de groei en ontwikkeling van immuun- en neuronale cellen beïnvloeden. Deze factoren werden bepaald in het serum van 64 gezonde vrouwelijke eerste of tweede graad familieleden van patiënten met een auto-immuun schildklierziekte. Van deze 64 vrouwen ontwikkelden 32 wel antistoffen en 32 geen antistoffen tegen TPO gedurende de 5 jaar van follow-up. Deze vrouwen werden vergeleken met 32 gezonde controles. Er werd een afwijkend gehalte vastgesteld in 4 groei- en differentiatiefactoren in de familieleden van patiënten met auto-immuun schildklierziekte, vergelijkbaar met de bevindingen in patiënten met stemmingsstoornissen. Dit suggereert dat dezelfde afwijkingen in groei en differentiatie van hematopoëse en neuronale cellen mogelijk ten grondslag liggen aan beide ziektebeelden.

In **hoofdstuk 5** wordt het verband beschreven tussen een uitgebreid schildklierhormoonprofiel en kwaliteit van leven in 143 patiënten met goed gedifferentieerd schildkliercarcinoom. Deze patiënten werden behandeld met een stabiele dosis van levothyroxine. De gezondheid-gerelateerde kwaliteit van leven werd met de RAND-36 vragenlijst bestudeerd, schildklier-specifieke kwaliteit van leven met de ThyPRO vragenlijst en vermoeidheid met de Multidimensionele Vermoeidheids Index. We constateerden dat gezondheid-gerelateerde kwaliteit van leven minder was in onze patiënten ten opzichte van een Nederlandse referentiegroep. We vonden in onze studiegroep echter geen associatie met diverse schildklierhormonen, inclusief 3,5-T2 concentraties en T4/T3 ratio's. Onze bevindingen

bieden dan ook geen bewijs dat het aanpassen van de dosering levothyroxine leidt tot verbetering van vermoeidheid en welbevinden.

Deel III: Zoektocht naar nieuwe biomarkers voor weefselspecifieke schildklierstatus

In het derde deel van dit proefschrift (**hoofdstuk 6 en 7**) wordt de zoektocht naar nieuwe biomarkers voor schildklierstatus beschreven. In **hoofdstuk 6** wordt onderzocht of microRNA's in serum worden gereguleerd door schildklierhormoon. Daarvoor werd de expressie van 384 microRNA's gemeten met een TaqMan MicroRNA Array in het serum van 13 patiënten met goed gedifferentieerd schildkliercarcinoom tijdens hypothyreoïdie en tijdens TSH-suppressie therapie. We vonden echter geen enkele significant verschillende microRNA.
In **hoofdstuk 7**, worden de resultaten van next-generation RNA sequencing in volbloedmonsters van een cohort van 8 patiënten met goed gedifferentieerd schildkliercarcinoom met en zonder behandeling met levothyroxine beschreven. Er werd aangetoond dat vele genen worden gereguleerd door schildklierhormoon. Aangezien we hebben bevestigd dat cellen in volbloed met name de schildklierhormoon receptor alpha (TRα) bevat reflecteren onze resultaten met name het effect van schildklierhormoon via TRα. We vonden een significante overlap met de voorheen gerapporteerde differentieel tot expressie komende genen in spiermonsters van patiënten met schildkliercarcinoom. Dit suggereert dat makkelijk toegankelijke volbloedmonsters gebruikt zouden kunnen worden als een model om het effect van schildklierhormoon op andere weefsels te bestuderen. Daarnaast vonden we ook dat schildklierhormoon de genexpressie in trombocyten (bloedplaatjes) bevordert. Dit zou een verklaring kunnen zijn voor de verhoogde tromboseneiging die is waargenomen in patiënten met hyperthyreoïdie.
In **hoofdstuk 8** tenslotte worden de resultaten van de diverse studies bediscussieerd en worden aanbevelingen gegeven voor toekomstig onderzoek.

Appendix

Acute Candida Thyroiditis Complicated by Abscess Formation in a Severely Immunocompromised Patient

Elske T. Massolt, Anita W. Rijneveld, Meike W. Vernooij, Marlies E. Kevenaar, Folkert J. van Kemenade, Robin P. Peeters

J Clin Endocrinol Metab (2014) 99 (11): 3952-3953.

A 33-year-old man was treated for acute lymphoblastic leukemia according to an intensive pediatric-inspired protocol with combination chemotherapy (1). At day 21 of his first chemotherapy course, he developed neutropenic fever. Blood cultures showed *Candida albicans*. The central venous catheter was removed, and iv fluconazole treatment was started for 14 days. At day 23, he developed a painful neck and palpitations. Ultrasound showed a diffusely enlarged hypoechogenic thyroid gland without nodules. Because of suspicion of a *Candida* thyroiditis, a fine-needle aspiration of the thyroid gland was performed, which revealed numerous yeast forms consistent with *Candida* (Figure 1), although cultures were negative. The treatment course with fluconazole was extended to 42 days. Thyroid function tests demonstrated hyperthyroidism (TSH = 0.012 mU/L; free T_4 > 90 pmol/L), converting within 2 weeks into hypothyroidism (TSH = 16.3 mU/L; free T_4 = 4.3 pmol/L). Levothyroxine treatment was then started and continued. At day 59, after bone marrow recovery, fever remitted in combination with a progressive painful cervical mass. Computed tomography showed a multiloculated abscess of the entire thyroid gland (Figure 2). Ultrasound-guided drainage was tried unsuccessfully, and ultimately surgical drainage of 250 mL of pus was performed, after which the abscess resolved. Blankophor staining demonstrated multiple fungal elements. *Candida* thyroiditis is a rare complication in immunocompromised patients with systemic *Candida* infections. Close monitoring is necessary to diagnose abscess formation in time (2). Microscopic examination of the fine-needle aspiration is a valuable diagnostic tool because cultures can remain negative, especially when systemic therapy has already been started (3, 4). Thyroid function should be closely monitored.

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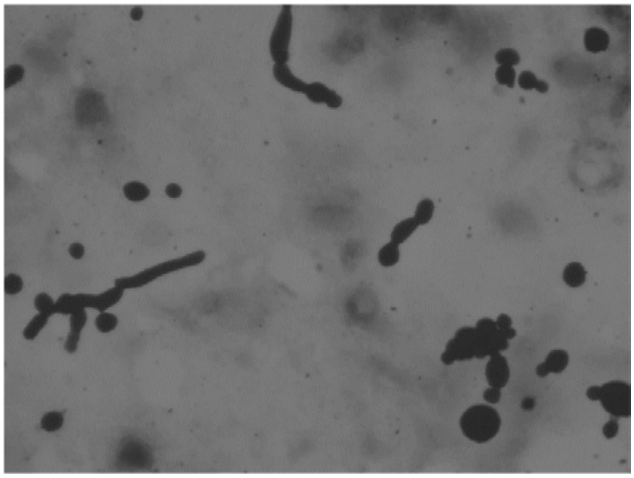

Figure 1. Grocott staining of the fine-needle aspiration showing yeast (magnification, ×40).

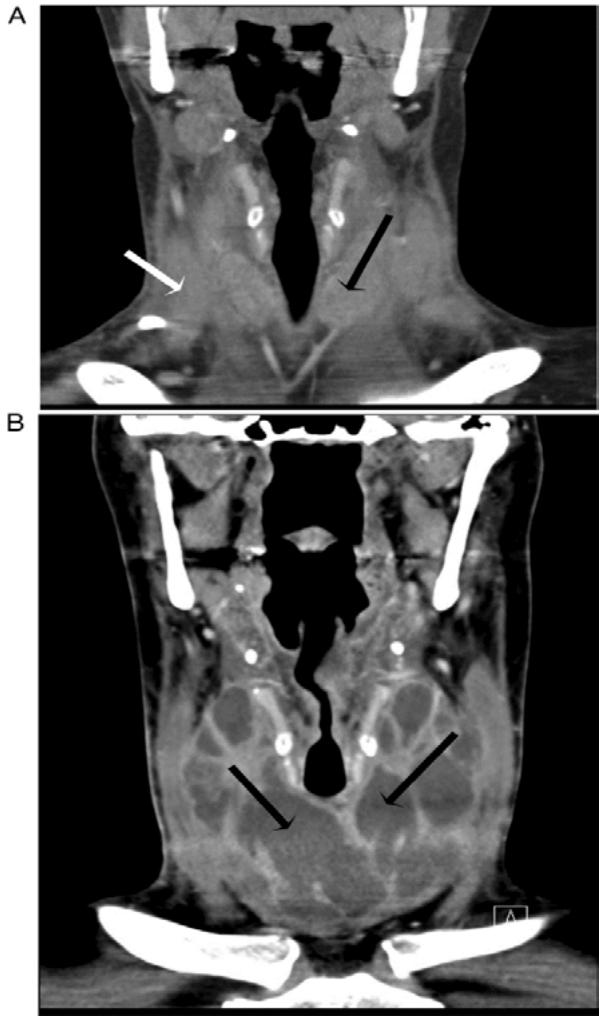

Figure 2. A, Computed tomography (CT) of the neck during an episode of neutropenic fever shows diffuse enlargement of the thyroid gland (black arrow) and edematous swelling of soft tissues (white arrow). B, CT scan 3 weeks later showing multiple low-density areas (arrows) representing a large multiloculated abscess of 100 × 60 × 100 mm within the thyroid gland.

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Congress visits: oral presentations	Year	ECTS
Dutch Endocrine Meeting, Noordwijkerhout	2015	0.8
Dutch Endocrine Meeting, Noordwijkerhout	2016	0.8
European Thyroid Association, Copenhagen	2016	0.8

Congress visits: poster presentations		
Science Days Internal Medicine, Antwerp	2015	0.8
International Thyroid Congress, Orlando	2015	0.8
Science Days Internal Medicine, Antwerp	2016	0.8
Annual meeting Endocrine Society, Boston	2016	0.8

Congress visits: other		
Annual meeting endocrine society, San Francisco	2013	0.8
European Thyroid Association, Leiden	2013	0.8
Science Days Internal Medicine, Antwerp	2014	0.6
European Thyroid Association, Santiago de Compostela	2014	0.8

Teaching activities		
Lectures on thyroid (dys)function, first year medical students	2013-2015	0.7
Lectures on adrenal (dys)function, first year medical students	2013-2014	0.5
Lecture on thyroid (dys)function, nurse practitioners	2013	0.3
Lecture on thyroid (dys)function, general practitioners	2014	0.3
Supervising the research project of a medical student	2015	1.0
Consultant in clinical reasoning, medical master students	2015	0.5
Attending Endocrinology Lectures, Erasmus MC	2013-2016	1.5

Research skills

NIHES course biostatistical methods 1	2013	5.7
Basic excel workshop	2014	0.3
Basic thyroid course, ETA, Santiago de Compostela	2014	0.3
Biomedical scientific English writing	2015	3.0
NGS applications in molecular medicine	2015	0.6
Scientific integrity	2015	0.3
Basic course human genetics, MolMed	2015	0.5
Endocrine trainee day, endocrine society, Boston	2016	0.3
Peer reviews for Clinical Endocrinology (8 reviews)	2016, 2017	0.8

Clinical courses/ activities

Member of organizing committee Rotterdamse internistendag	2013	1.0
master class: RFA of benign thyroid nodules, Trieste	2014	0.6
Rotterdamse internistendag	2014	0.3
Annual Dutch symposium endocrinology, Utrecht	2015, 2017	0.4
DOO: hospital management	2015	0.5
Course on medically unexplained physical symptoms, Erasmus MC	2016	0.6
Tumor board meetings	2013-2016	3.0
Subcommittee regional thyroid network "zorgpad schildkliernodi"	2016	1.0
DESG (diabetes) course for fellows in endocrinology	2016	0.3

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About the author

Elske Massolt was born on May 19th, 1979 in Witmarsum. After completing secondary school at the R.S.G. Simon Vestdijk in Harlingen, she moved to Groningen to study Pedagogy and Educational Sciences at the university of Groningen (RUG). After finishing her first year, she started her study of Medicine at the Erasmus University Rotterdam in 1999. In November 2005 she received her medical degree and started as a resident (ANIOS) in Internal Medicine at the Medical Center of The Hague (MCH). In May 2007, she continued her residency (AIOS) at the Reinier de Graaf hospital of Delft under supervision of Dr. E. Maartense and Dr. E.F. Posthuma, and from the beginning of 2010 at the Erasmus MC under supervision of Prof.dr. J.L.C.M. van Saase. In 2011, she started her fellowship in Endocrinology at the Erasmus MC under supervision of Prof.dr. W.W. de Herder. In May 2013 she started as a PhD-student in the thyroid center of Prof.dr. R.P. Peeters and the results of her 3 years of PhD research are presented in this thesis. She received a travel grant award from the Endocrine Society (ENDO 2016, Boston, USA) and she won the 2015 Trainee Poster Contest at the 15th International Thyroid Congress (Orlando, USA). In June 2016 she started as an internist-endocrinologist at the Albert Schweitzer hospital of Dordrecht. She married Marnix Muller in 2008 and they have two fantastic children Benthe and Tieme.