Intestinal Absorption of Thyroid Hormone

Nienke Kelderman-Bolk

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De opname van schildklierhormoon in de darm

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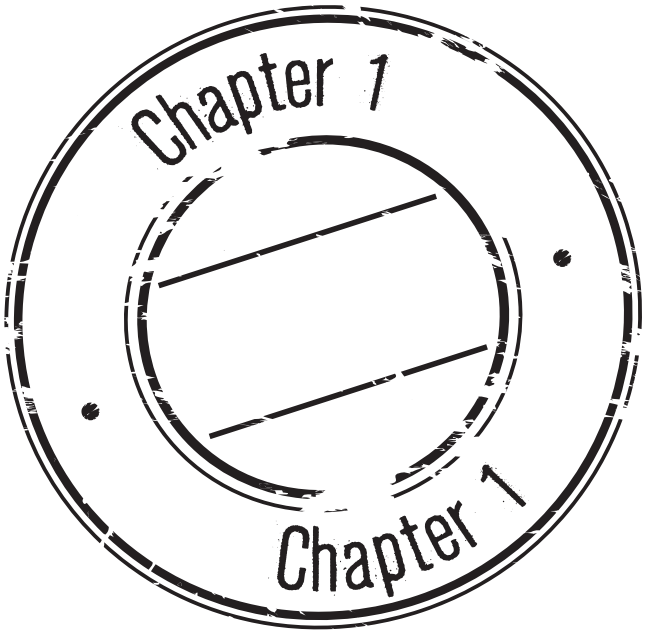
Contents



General Introduction

INTRODUCTION

Thyroid hormone is important in virtually all metabolic processes in the human body. Deficiency of thyroid hormone (hypothyroidism) results in fatigue, weight gain, cold intolerance and constipation, and may eventually lead to depression, hypertension and atherosclerosis. The synthetic thyroid hormone levothyroxine (LT4) is the treatment of choice to reduce these symptoms. In clinical practice, however, adequate treatment is not always easy to achieve.

This thesis originally started with a clinical observation in a hypothyroid patient on LT4 treatment with persistent complaints. The research went from bedside to bench and focused on the uptake of the thyroid hormone T4 by the intestine. We studied the effect of switching from morning LT4 intake to bedtime LT4 intake on thyroid hormone levels and profiles. Subsequently, the quality of life (QOL) in treated hypothyroid patients was studied. Thyroid hormone transport in the enterocyte (small intestinal cell) was characterized in an intestinal cell model. The results of these basal experiments provided important information, offering more understanding of reduced absorption of LT4 in several clinical settings.

HYPOTHALAMUS-PITUITARY-THYROID AXIS

Thyroid hormone is produced by the thyroid gland, which is located in the neck. The production of thyroid hormone is regulated by the hypothalamus-pituitary-thyroid axis (Figure 1). Thyrotropin releasing hormone (TRH), produced by the hypothalamus, stimulates thyrotropin (thyroid stimulating hormone, TSH) secretion by the anterior pituitary. By stimulating the TSH receptor, TSH activates the thyroid gland to produce thyroid hormones. In the follicular cells of the thyroid gland the important element iodide is incorporated into thyroglobulin by thyroperoxidase (TPO), followed by the production of mono- and diiodothyrosines. TPO also catalyses the coupling of these iodothyrosines to form 3,3’,5-triiodothyronine (T3) and 3,5,3’,5’-tetraiodothyronine (thyroxine, T4). Finally, the iodothyronines are released into the circulation. In humans, the thyroid gland predominantly produces the inactive prohormone T4 and a small amount of the active hormone

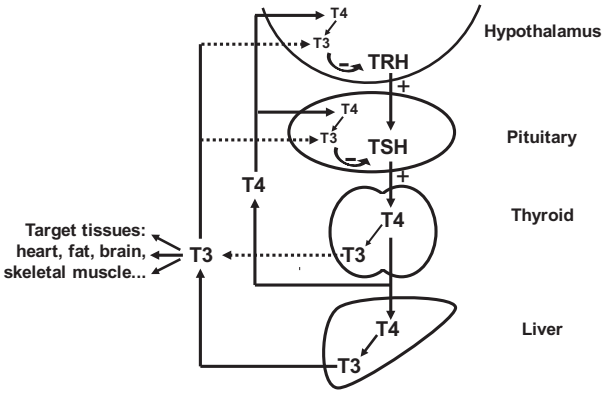


Figure 1. The hypothalamus-pituitary thyroid axis

triiodothyronine (T3). 80% of the circulating T3 is produced outside the thyroid by peripheral conversion of T4 by the iodothyronine deiodinases type 1 and 2 (D1 and D2). D1 is present in liver and kidney, and D2 is mainly present in brain, brown adipose tissue, skeletal muscle and heart. Because thyroid hormone gives negative feedback to the hypothalamus and pituitary gland, the production of TRH and TSH can be downregulated. This ascertains that serum thyroid hormone levels are maintained within a narrow range.

HYPOTHYROIDISM

Hypothyroidism is the lack of thyroid hormone in the serum, resulting in a generalized slowing of metabolic processes in the body. The cause of the reduced thyroid hormone production can be located in the thyroid gland itself (primary hypothyroidism), in the pituitary gland (secondary hypothyroidism) or in the hypothalamus (tertiary hypothyroidism). In primary hypothyroidism T4 and T3 levels are low, causing the pituitary gland to produce high levels of TSH to stimulate the thyroid gland. In secondary and tertiary hypothyroidism the TSH production is low (or inappropriately normal) resulting in low serum thyroid hormone levels. This thesis will mainly focus on the treatment of primary hypothyroidism.

There are several causes of inadequate production of thyroid hormone by the thyroid gland. Congenital hypothyroidism is a lack of thyroid hormone at birth, usually because the thyroid gland has not developed during fetal growth. Since the introduction of postnatal screening in the Netherlands this condition is diagnosed shortly after birth. Prompt initiation of LT4 treatment after diagnosis, prevents the development of cretinism (mental retardation and impaired growth) in these children.

Environmental iodine deficiency is the most common cause of hypothyroidism on a worldwide basis: iodide is an essential element in thyroid hormone production. In areas of iodide sufficiency, like the Netherlands, the most common cause of hypothyroidism is autoimmune thyroiditis (Hashimoto's thyroiditis). It is characterized by circulating thyroid autoantibodies, which include anti-thyroid peroxidase antibodies (TPOAb), anti-thyroglobulin antibodies (TgAb) and TSH receptor antibodies (TSHRAb).

Hypothyroidism may also occur after radioiodine or surgical treament for hyperthyroidism, thyroid cancer or benign nodular thyroid disease and after external beam radiation for head-and-neck related malignancies. Finally, certain drugs such as lithium (1), amiodarone (2) and iodide excess (3) can induce hypothyroidism that requires treatment. Recently, tyrosine kinase inhibitors, most notably sunitinib, have been discovered to cause hypothyroidism as well (4).

TREATMENT OF HYPOTHYROIDISM

The treatment of hypothyroidism has only been possible since the end of the 19th century. After the anatomical identification of the thyroid gland in the neck in 1656, the function of the gland remained unresolved for another 200 years (5). It was considered a rudimentary structure, with no specific function in the adult human. In 1883 the Swiss physician Theodor Kocher presented a paper at the XII. Congresses der Deutschen Gesellschaft fur Chirurgie zu Berlin describing his patients after thyroid surgery because of mechanical problems caused by struma (6). After a remarkable deterioration in the condition of one of his patients postoperatively, he called to follow up 101 patients he had performed thyroid surgery on. In those cases where he had performed a total

thyroidectomy he concluded that symptoms were similar to patients with cretinism. This observation resulted in the hypothesis that an abnormally low secretion of thyroid hormone from the thyroid gland (hypothyroidism), could lead to a decrease in basal metabolic rate and cause symptoms of fatigue, weight gain and cold intolerance.

In 1891 Murray (United Kingdom) first described treatment of a human patient with hypothyroidism with subcutaneously injected sheep thyroid extract, which helped to resolve most symptoms (7). Soon after, it was discovered that oral administration of thyroid extract was effective. Animal-derived dessicated thyroid gland (Thyranon in the Netherlands) was the treatment for hypothyroidism for the next decades. However, the content of thyroid hormone varied in dessicated thyroid hormone preparations, and contained supraphysiologic levels of T3. Purified thyroxine (T4) was discovered in 1914, and in 1930 synthetic thyroxine was developed. By the 1960s, it was known that thyroxine was the essential hormone produced by the thyroid gland, and that most T3 was produced at tissue level by deiodination of thyroxine. In the Netherlands, synthetic thyroxine, levothyroxine (LT4), became available in the 1970s when the pharmaceutical company Organon introduced Thyrax. After intake, LT4 has a half-life of 6 days, thus providing the body with stable and physiological quantities of T3 (8, 9). International guidelines therefore recommend LT4 monotherapy as the best substitution therapy in hypothyroidism (10). Over the years the prevalence of hypothyroidism has increased, with now 450 thousand people taking LT4 in the Netherlands (Source: GIP/Zorginstituut Nederland, update 07-03-2014).

ADEQUATE TREATMENT OF HYPOTHYROIDISM

Adequate replacement therapy of LT4 is defined by reaching normal free T4 (FT4) and thyrotropin (TSH) levels. As there is a negative feedback of T4 and T3 on TSH secretion by the pituitary gland, TSH is a good marker of peripheral thyroid hormone action. Therefore, TSH is the most reliable marker of adequate replacement treatment in the follow up of hypothyroid patients. To reach stable TSH and thyroid hormone levels, an adequate uptake of LT4 by the small intestine is essential. As absorption is decreased by several drugs and simultaneous food intake

(11, 12), Dutch prescribing information states that LT4 should be taken 30 minutes before breakfast. American prescribing information advises an interval of 60 minutes before taking any food. In addition, interfering drugs should be taken 2-6 hours after LT4 ingestion (11). For patients these instructions can be inconvenient because of their (work)schedule, concurrent illnesses (eg diabetes) and other drugs that should be taken before or with meals.

We observed several patients whose thyroid hormone levels and symptoms improved when LT4 was taken at bedtime instead of in the morning (following the patient information leaflet). One illustrative case is described in the frame on this page. These observations made us wonder if LT4 at bedtime was as good, or even better, than LT4 ingestion in the morning. A review of the literature showed that it had never been studied systematically whether intestinal LT4 absorption is better when the stomach is empty in the morning than at night.

CLINICAL CASE

A 45 year old hypothyroid male patient, who happened to be a pharmacist, was referred to our outpatient clinic for complaints of fatigue. Since diagnosis, several years before, he had been treated with LT4, in a dosis of 150 µg daily (1.8 µg/kg) The laboratory values indicated inadequate substitution of the hypothyroidism (TSH 11.8 mU/l (normal 0.4- 4.0 mU/l) and FT4 18.4 pmol/l (normal 10-24 pmol/l). As he reported to take the thyroid hormone tablet first thing in the morning, we suggested to take the tablet at bedtime. At the return visit after 6 weeks, he reported a significant improvement in well being. Interestingly, his TSH level was also normalized (TSH 3.5 mU/l, FT4 21.0 pmol/l).

THYROXINE INTAKE AT BEDTIME

Because of the clinical observation described above, we decided to study the effect of bedtime intake of LT4 on thyroid hormone levels and quality of life (QOL). The optimal study design to investigate morning versus bedtime LT4 intake was considered to be a randomized double blind trial. However, the best time for blood collection in such a trial could not easily be determined. Some reports have mentioned acute changes in FT4 levels following ingestion of LT4 (13). Furthermore, TSH has a circadian rhythm (14), which might be influenced by altering the administration time of LT4. Before initiating the randomized trial, we decided, therefore, to study the effect of bedtime LT4 intake on TSH and thyroid hormone levels and their circadian rhythm.

Circadian rhythm thyroid hormones

Various hormones in the human endocrine system exhibit a circadian rhythm, facilitating anticipation of and adaptation to environmental fluctuations (15). Cortisol, for example, peaks during the morning and prepares the body for typical stresses associated with wakening. Thyrotropin (TSH), released by the pituitary gland, also exhibits a circadian rhythm. Serum TSH levels increase in the evening, reach a maximum in the early night and are followed by a progressive decrease during the night and low levels during the day (14, 16). TSH secretion is influenced by thyrotropin releasing hormone (TRH), negative feedback of thyroid hormones and various other factors (17). The circadian rhythm is still present in mild hypothyroidism, disappears in overt hypothyroidism, but is restored with LT4 substitution (18). There are conflicting data on a diurnal rhythm in T4 and T3 levels (19, 20). Some studies show that T4 rises after LT4 ingestion. The influence of the time of LT4 ingestion on circadian patterns of thyroid hormones is unclear, and changing the administration time of LT4 to bedtime in patients with primary hypothyroidism might influence the nocturnal TSH surge and the circadian variation of serum TSH. In that case, taking one blood sample per day may not represent average hormone levels, as this single blood sample may be drawn on different moments of the hormone cycles. **Chapter 2** describes the 24 hour patterns of thyroid hormones with morning and bedtime LT4 intake in 11 patients (21). The aim of this study was to confirm the clinical observation of improvement of thyroid hormone levels when switching to bedtime LT4, and to determine the optimal time for blood sample collection in a future randomized trial.

Morning versus evening LT4 intake

As the pilot study in 11 patients showed significant differences in thyroid hormone levels, we wanted to confirm these findings in a randomized double blind cross over trial on morning versus bedtime LT4 ingestion. The pilot study had also shown that blood sampling could take place in the morning as usual. **Chapter 3** describes the trial including 90 consecutive patients of our outpatient clinics treated for hypothyroidism. During the first 3 months patients took LT4 in the morning and placebo at night and switched to bedtime LT4 and morning placebo for another 3 months (or vice versa). Thyroid hormone levels and vital parameters were checked every 6 weeks. We also studied the quality of life (QOL), often mentioned by hypothyroid patients to be diminished. Therefore, the patients completed 3 QOL questionnaires and a symptom score at 0, 3 and 6 months. The cross over design enabled us to compare QOL between morning and bedtime LT4 intake, with patients being their own controls.

REDUCED QOL IN HYPOTHYROIDISM

Previous trials have shown that despite normalization of serum thyroid hormones by suppletion with LT4, many patients with treated primary hypothyroidism still complain of a reduced QOL (22, 23). The reason for this reduced QOL has not been completely elucidated. As symptoms of hypothyroidism improve after starting LT4 replacement therapy, it seems plausible to assume that there is a correlation between thyroid hormone parameters and QOL. Attempts to improve QOL by aiming at a lower serum TSH level (24, 25), by adding liothyronine (T3) substitution to thyroxine (26), or by making small changes in the LT4 dose (27), have not resulted in improvement in well-being. Of course the narrow individual setpoint for thyroid hormone levels makes it difficult to find the right substitution dose for individuals (28). Another aspect in the treatment of hypothyroidism is that serum levels may not represent thyroid hormone levels in different tissues (29). A marker for thyroid hormone tissue levels would be helpful to improve treatment.

More knowledge on the reason of reduced QOL in hypothyroid patients, would help to have a better understanding of patients' complaints and to optimise their treatment. In **Chapter 4** we analyse the correlation between QOL and the various measured parameters in our randomized

trial. The correlation of thyroid hormone levels, vital parameters and autoimmune antibodies and QOL is described.

THYROID HORMONE UPTAKE IN THE GUT

While we were conducting the clinical studies on LT4 administration, we gathered the available information on the factors influencing LT4 uptake by the intestine. Apparently, the mechanism of thyroid hormone uptake in the small intestine had not been completely cleared up. We contacted the research laboratory of the department of Endocrinology at the Erasmus Medical Centre, which has great expertise in research on thyroid hormone transport (30), and decided to perform experiments to characterise thyroid hormone transport in intestinal cells.

Previous studies had shown that uptake of LT4 takes place in the duodenum, jejunum and ileum, and is approximately 70-80% (31). As thyroid hormones have to enter the cell to be metabolised by deiodinases and to activate the nuclear thyroid hormone receptor, transport of thyroid hormone over the cell membrane is required. Based on the lipophilic structure of thyroid hormones, it was previously assumed that thyroid hormones enter the cell through passive diffusion (31, 32). However, experimental evidence and clinical studies over the last decades have shown clearly that thyroid hormones traverse the cell membrane mainly through transporters (33, 34). Thyroid hormone uptake has different characteristics across cell types, with regard to ligand specificity, energy (ATP) dependence, Na^+-dependence and interactions with a variety of compounds (33). It has become apparent that different transporters facilitate thyroid hormone uptake in different tissues. Over the last years several thyroid hormone transporting proteins have been identified. These include Na^+/tauchlorate cotransporting polypeptide (35, 36), fatty acid translocase (37), multidrug resistance-associated proteins (38), amino acid transporters (39) and members of the organic anion-transporting polypeptide (OATP) family (40). It has, however, not been clarified up till now which transporter is responsible for thyroid hormone uptake by the intestinal cell. This made us look for a cell line to study the characteristics of thyroid hormone uptake in the small intestine. Experiments with enterocytes are not possible, as intestinal biopsies contain different cell types and are contaminated with the intestinal flora. The intestinal model

Caco2 (colon carcinoma) is a cell line that in vitro undergoes spontaneous enterocytic differentation. This makes it a reliable in vitro model to study intestinal absorption, transport and metabolism (41). In **Chapter 5** we provide evidence that there is transport of T3 and T4 in Caco2 cells and describe the transport characteristics.

FACTORS INFLUENCING LT4 ABSORPTION

The studies on the best time of LT4 intake and the experiments on T4 uptake by intestinal cells, made it clear that there are many factors influencing the absorption of T4. The absorption can be decreased by food, beverages, gastrointestinal disorders and several drugs. In **Chapter 6** we give an overview of the latest available literature on this subject. Guidelines are given to ensure optimal absorption of LT4. When patients have a persistent elevation of TSH levels, despite high doses of LT4, it is emphasized that malabsorption by gastrointestinal disorders or drugs should be excluded. Finally, pseudo-malabsorption (non-compliance) is often a diagnostic challenge. A guideline is presented how to carefully investigate this clinical problem.

IN PERSPECTIVE

In **Chapter 7** we discuss the implications of our clinical studies on daily practice. We describe the (inter)national guidelines that have included bedtime LT4 intake as an alternative to morning intake. Suggestions for further research on QOL in hypothyroidism are posted.

The results of the experiments on T4 and T3 uptake in Caco2 cells are discussed, and suggestions on further research are made.

SUMMARY

Chapter 8 and 9 are the English and Dutch summary of this thesis, respectively.

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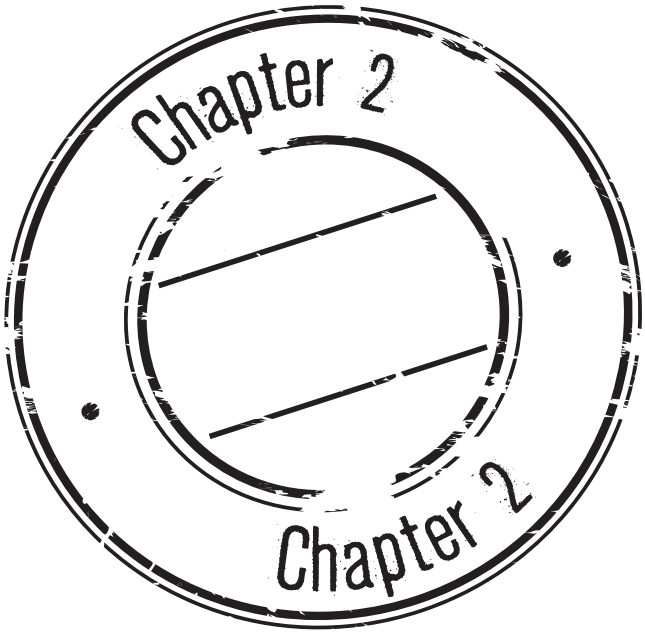

Effects of Evening versus Morning Thyroxine Ingestion on Serum Thyroid Hormone Profiles in Hypothyroid Patients

Nienke Bolk
Theo J Visser
Andries Kalsbeek
Ron T van Domburg
Arie Berghout

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SUMMARY

Objective Standard drug information resources recommend that L-thyroxine be taken half an hour before breakfast on an empty stomach, to prevent interference of its intestinal uptake by food or medication. We observed cases in which TSH levels improved markedly after changing the administration time of L-thyroxine to the late evening. We therefore conducted a pilot-study to investigate whether L-thyroxine administration at bedtime improves TSH and thyroid hormones, and whether the circadian rhythm of TSH remains intact.

Design Patients were studied on two occassions: on a stable regimen of morning thyroxine administration and two months after switching to night-time thyroxine using the same dose. On each occasion patients were admitted for 24 h and serial blood samples were obtained.

Patients We investigated 12 women treated with L-thyroxine because of primary hypothyroidism, who used no medication known to interfere with L-thyroxine uptake.

Measurements Patients were admitted to hospital and blood samples were obtained at hourly intervals for 24 h via an indwelling catheter. Following this first hospital admission, all women were asked to switch the administration time from morning to bedtime or vice versa. After 2 months they were readmitted for a 24-h period of hourly blood sampling. Blood samples were analysed for serum TSH (immunometric assay), FT4 and T3 (competitive immunoassay), T4 and rT3 (radioimmunoassay), serum TBG (immunometric assay) and total protein and albumin (colourimetric methods).

Results A significant difference in TSH and thyroid hormones was found after switching to bedtime administration of L-thyroxine. Twenty-four-hour average serum values amounted to (mean ±SD, morning *vs* bedtime ingestion): TSH , 5.1 ± 0.9 vs 1.2 ± 0.3 mU/l ($p < 0.01$); FT4, 16.7 ± 1.0 vs 19.3 ± 0.7 pmol/l ($p < 0.01$); T3, 1.5 ± 0.05 vs 1.6 ± 0.1 nmol/l ($p < 0.01$). There was no significant change in T4, rT3, albumin and TBG serum levels, nor in the T3/rT3 ratio. The relative amplitude and time of the nocturnal TSH surge remained intact.

Conclusions L-thyroxine taken at bedtime by patients with primary hypothyroidism is associated with higher thyroid hormone concentrations and lower TSH concentrations compared to the same L-thyroxine dose taken in the morning. At the same time, the circadian TSH rhythm stays intact. Our findings are best explained by a better gastrointestinal uptake of L-thyroxine during the night.

INTRODUCTION

Levothyroxine is currently one of the most prescribed medications (1). For the widely used oral preparations, enteral absorption of L-thyroxine is approximately 70-80% (2-4). The small bowel is the primary site of thyroid hormone absorption by a mechanism of translocation across the mucosa that remains unclear (5). Interference with L-thyroxine absorption has been documented for cholestyramine resin, colestipol hydrochloride, sucralfate, iron sulfate, aluminum antacids, activated charcoal, raloxifen, food and herbal remedies (2,6). Also, a fiber-enriched diet has been shown to have an adverse effect on the intestinal absorption of L-thyroxine (7). Therefore, standard drug information resources, including manufacturers' prescribing information, recommend that L-thyroxine be taken on an empty stomach in the morning. Consequently, hypothyroid patients worldwide are advised to take L-thyroxine tablets in the morning half an hour before breakfast. However, whether intestinal absorption is better when the stomach is empty in the morning or at night has never been studied systematically. Recently, we observed several patients whose thyroid hormone profiles improved markedly after changing the administration time of L-thyroxine to bedtime. This prompted us to further study this phenomenon.

The circadian variation of serum TSH in man is well-documented. The serum levels of TSH increase in the evening, reach a maximum near sleep onset and are followed by a progressive decrease during the night and low values during the day (8-11). The percentage nocturnal rise of TSH is 71 ± 40% in healthy controls, and is maintained in euthyroid patients on levothyroxine therapy taken in the morning (63 ± 51%) and patients with mild hypothyroidism (54 ± 33%) (10), whereas in overt hypothyroidism this nocturnal surge disappears.

Changing the administration time of L-thyroxine to bedtime in patients with primary hypothyroidism might influence the nocturnal TSH surge and the circadian variation of serum TSH. In that case, taking one blood sample per day may not represent average hormone levels, as this single blood sample may be drawn on completely different moments of the hormone cycles.

Thyroid hormone deiodination by deiodinases type 1, 2 and 3, and other pathways of T4 metabolism could also be affected by altering the administration time of L-thyroxine.

The aim of this pilot study was, to investigate the effect of changing the administration time of L-thyroxine from early morning to bedtime on (1) thyroid hormone profiles, (2) the circadian rhythm of TSH and thyroid hormones, and (3) thyroid hormone metabolism.

PATIENTS AND METHODS

Twelve women, aged 25-75 yrs (mean 48 yrs), on L-thyroxine treatment because of primary hypothyroidism, volunteered for this study. The mean dose of L-thyroxine (Thyrax, Organon) was 121 µg, the mean body weight 78 kg. None of the patients used medication known to interfere with L-thyroxine absorption, nor were they known to have gastro-intestinal disease. The cause of hypothyroidism was primary (auto-immune) hypothyroidism in 8 subjects; in 3 subjects previous radioiodine treatment given because of Graves' disease and in one thyroidectomy because of nodular goitre. All subjects gave written consent and the protocol was approved by the local ethics committee.

Patients were admitted to the hospital for 24 h on two separate days, with a two-month interval. On the first day all subjects were still taking L-thyroxine at their usual time (10 women in the morning, 2 women at bedtime). The former were used to taking L-thyroxine half an hour before breakfast, between 6:00 and 7:00 a.m. Blood samples were obtained at hourly intervals for 24 h via an indwelling catheter, followed by rinsing with heparinized saline.

Following this first hospital admission, all women were asked to switch the administration time from morning to bedtime at 10:00 p.m. (and vice versa from bedtime to morning between 6 and 7 a.m.). After 2 months they were admitted again for a 24-h period of hourly blood sampling. During admission all subjects were ambulant during the day, meals were taken at standardized times (7:30 a.m., 12:30 p.m. and 5:30 p.m.)**,** and they were in bed from 11.00 p.m. to 7.00 a.m.

Blood samples were immediately centrifuged and stored at 4 °C. Within 12 h the plasma samples were divided in small aliquots and stored at –80 °C until analysis. Serum TSH (normal range 0.4-4.0 mU/l) was measured by immunometric assay (Immulite 2000), with a detection limit of 0.002 mU/l. The interassay variation was 12.5, 4.6, 5.1, 4.5 and 6.4% at mean TSH values of 0.02, 1.3, 7.3, 19.0 and 39.0 mU/l, respectively. FT4 (normal range 10.0-24.0 pmol/l) and T3 (normal range 1.23-2.80 nmol/l) were measured by competitive immunoassay (Immulite 2000), T4 (normal range 58-128 nmol/l) and rT3 (normal range 0.14-0.34 nmol/l) by radioimmunoassay, serum TBG (normal range 12-38 mg/l) by immunometric assay (Immulite 2000), and total protein and albumin by colorimetric methods.

Statistical analysis was carried out using ANOVA, paired student t-tests and paired-samples t-test for the blood pressure and pulse frequency. Significance of diurnal variation (time dependency) of the different hormone curves was assessed using a one-way ANOVA with repeated measures. Only if ANOVA detected a significant effect of time, a cosinor analysis was performed on the data sets of individual patients, using the fundamental period (24h). Curve fitting was performed using constrained nonlinear regression analysis (SPSS 11.0). Subsequently, only if the significance level of the fitted curve was less than 0.05, data were used to calculate circadian rhythm parameters (12). Student's t-test (paired) were used to detect significant differences between the curves of the morning and evening treatment. For the ANOVA, (paired) t-tests and the cosinor analysis, $p<0.05$ was considered to be a significant difference. In all cases, statistics and cosine analysis were done on absolute values. Circular statistics were applied for the acrophase data, that is, Jupp's Phi and S for mean angle and angular standard deviation, Mardia–Watson–Wheeler Chi-square test for evaluation of acrophase differences between groups (13). This was not a randomized double-blind study although hormone measurements and statistical analyses were done without information about the time of L-thyroxine ingestion.

We asked all patients to fill out forms concerning symptoms of hypothyroidism and Quality of Life (QOL) during the periods of morning administration and bedtime administration. Firstly, a subjective symptom questionnaire which assessed 20 symptoms of hypothyroidism and/or hyperthyroidism (for example depression, weight gain, cold/heat

intolerance, constipation etc.) was obtained (14). Patients scored these symptoms as not present (1 point), hardly present (2 points), present (3 points), or severely present (4 points). Secondly, a general QOL questionnaire (RAND-36) (15) was used to measure health related QOL according to 8 subscales. These subscales are: physical functioning, role physical functioning, bodily pain, general health perception, role emotional functioning, emotional well-being, energy/fatigue and social functioning. The scale score ranged from 0-100 for every subscale, with a higher outcome meaning a better health status.

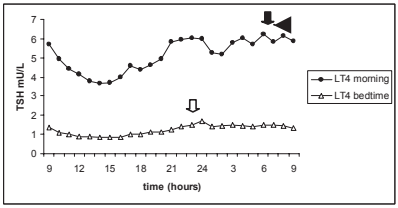
RESULTS

Serum TSH levels decreased and FT4 and T3 levels increased remarkably and significantly after changing the time of L-thyroxine ingestion from morning to bedtime (Figure 1). The results of 11 patients are given, as it was impossible to gain venous access in one subject. Table 1 shows all the 24-hours mean values ± standard deviation for TSH, FT4, T3, T4, rT3, the T3/rT3 ratio, albumin and TBG during morning and bedtime thyroxine administration.

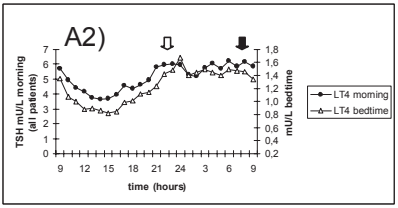
When levothyroxine is taken in the morning there is an increase in serum FT4 after taking the tablet **(**$p<0.05$**),** which is not found after taking the tablet at bedtime, as is shown in Figure 1B. Serum rT3 levels, the biologically inactive product of inner ring deiodination of T4, did not change significantly, and neither did TT4, with time of thyroxine digestion. The ratio of active versus inactive thyroid hormone (T3/rT3), a parameter which reflects thyroid hormone metabolism by outer ring versus inner ring deiodination did not differ between the morning or bedtime ingestion of levothyroxine.

TSH decreased and FT4 rose in all patients by changing thyroxine ingestion from early morning to bedtime and T3 levels rose in all but one subject. A decrease in TSH levels was observed irrespective of initial TSH levels. The 24-h mean TSH levels during both the morning and bedtime administration of levothyroxine are negatively correlated with the 24-h mean FT4 levels ($R= -0.64$).

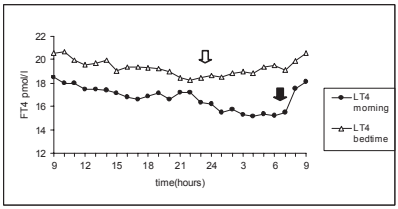
A1)

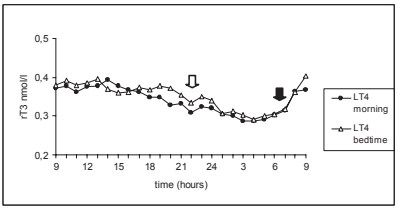

TSH mU/L
time (hours)
LT4 morning
LT4 bedtime

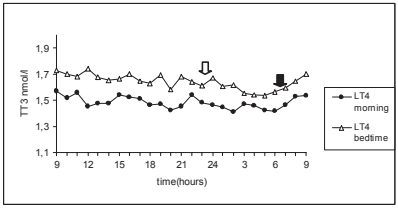
A2)

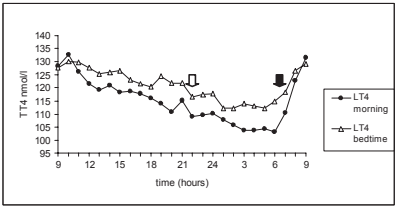

A2)
TSH mU/L morning (all patients)
mU/L bedtime
time (hours)
LT4 morning
LT4 bedtime

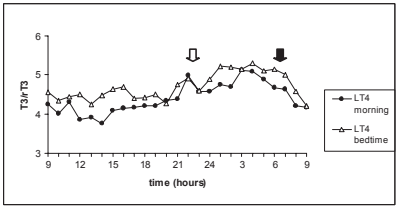
B)

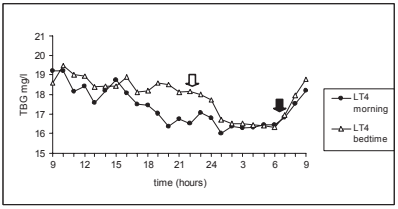

FT4 pmol/l
time(hours)
LT4 morning
LT4 bedtime

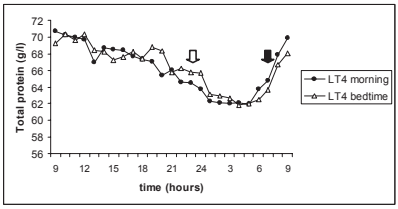

rT3 nmol/l
time (hours)
LT4 morning
LT4 bedtime


TT3 nmol/l
time(hours)
LT4 morning
LT4 bedtime


TT4 nmol/l
time (hours)
LT4 morning
LT4 bedtime


T3/rT3
time (hours)
LT4 morning
LT4 bedtime


TBG mg/l
time (hours)
LT4 morning
LT4 bedtime


Total protein (g/l)
time (hours)
LT4 morning
LT4 bedtime

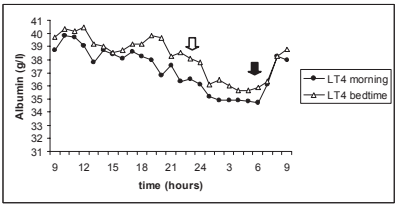

Albumin (g/l)
time (hours)
LT4 morning
LT4 bedtime

Figure 1.
Thyroid hormones and TSH during the morning administration of levothyroxine (●) and the bedtime administration of levothyroxine (Δ). Levothyroxine was taken at 7.00 a.m. ↓ or at 10.00 p.m ⇩. A1) Mean TSH serum levels of all patients over 24 hours. On the same scale. A2) TSH levels on different scales (morning administration on the left axis, bedtime administration on the right axis) to show that the circadian rhythm of TSH stayed intact during the morning and bedtime administration. B) Mean FT4, TT3, rT3, TT4, TBG, total protein, albumin serum levels and the T3/rT3 ratio of all patients over 24 hours.

Table 1
Difference in serum levels of thyroid hormones and their binding proteins, between the morning administration and bed time administration of levothyroxine. Data given as the *24 hours* mean hormonal values ± standard deviation of all 11 patients.

	TSH mU/l	**FT4 pmol/l**	**TT3 nmol/l**	**TT4 nmol/l**	**rT3 nmol/l**	**TT3/rT3**	**Albumin g/l**	**TBG Mg/l**
LT4 Morning	5.1±0.9	16.7±1.0	1.48±0.05	115.3±8.8	0.34±0.03	4.4±0.38	37.2±1.6	17.3±1.0
LT4 Bedtime	1.2±0.3	19.3±0.7	1.64±0.1	121.2±6.0	0.35±0.03	4.70±0.34	38.2±1.6	17.9±1.0
p-value	<0.01	<0.01	<0.01	NS	NS	NS	NS	NS

The pattern of the circadian rhythm stayed intact during the morning and bedtime administration of levothyroxine, and is almost identical (Figure 1, A2). The ANOVA and cosinor analysis showed a significant effect of time on all parameters (Table 2) except for T3 (not shown). FT4 as well as T4 show a significant difference between the morning administration and the bedtime administration in the 24-h mean values of the fitted curves, with higher values after the bedtime administration. In addition, bedtime administration slightly decreased the relative amplitude of their daily rhythms and phase-advanced the timing of their acrophase. Daily TSH rhythms were not affected by the timing of L-thyroxine intake.

The mean blood pressure was 130/75 mmHg during the morning dosage and 127/77 mmHg during the bedtime dosage (NS) and mean pulse rate 68/min and 65/min, respectively (NS).

The subjective symptom questionnaire showed no change in quality of life, with 41.5 ± 9.9 points during the morning administration of

Table 2
Results of cosinor analysis for significance of diurnal variation. Only if ANOVA detected a significant effect of time, a cosinor analysis was performed on the data sets of individual patients (n = the number of patients showing a significant fit). Only if the significance level of the fitted curve was less than 0.05 data were used to calculate circadian rhythm parameters. The fitted function is defined by its mesor (rhythm-adjusted mean), amplitude (50% of the difference between the maximum and the minimum of the fitted curve, expressed as a percentage of the mesor), and acrophase (time of the maximum). R^2, goodness of fit; A, morning administration; B, bedtime administration. P-values indicate the result of the paired student t-test on the means of A and B. Significant differences are indicated in bold.

	Amplitude	R^2	Mesor	Acrophase	n
TSH					
A	39.2 ± 3.6	74 ± 4%	5.6 ± 2.5	02:20 ± 1:25	11
B	34.9 ± 2.6	71 ± 3%	1.3 ± 0.4	02:25 ± 1:28	10
n	10	19	10	10	
p-value	0.144	0.51	0.082	0.19	
FT4					
A	9.8 ± 1.1	51 ± 6%	17.0 ± 0.8	13:34 ± 1:15	9
B	5.9 ± 0.7	44 ± 6%	20.2 ± 1.0	10:23 ± 2:05	9
n	8	8	8	8	
p-value	**0.04**	0.71	**0.006**	**0.04**	
Total protein					
A	6.7 ± 0.6	63 ± 5%	66.2 ± 0.9	12:50 ± 1:28	11
B	5.9 ± 0.8	48 ± 6%	66.4 ± 0.9	13:31 ± 1:35	11
n	11	11	11	11	
p-value	0.299	**0.013**	0.72	0.22	
Albumin					
A	6.7 ± 0.6	62 ± 6%	37.5 ± 0.6	13:51 ± 1:32	11
B	5.4 ± 0.8	54 ± 6%	38.4 ± 0.7	13:59 ± 2:18	10
n	10	10	10	10	
p-value	0.073	0.232	0.186	0.50	
rT3					
A	15.5 ± 2.0	58 ± 5%	0.35 ± 0.04	13:26 ± 1:06	11
B	15.0 ± 0.8	52 ± 5%	0.35 ± 0.04	14:09 ± 1:53	10
n	10	10	10	10	
p-value	0.580	0.367	0.840	0.98	

Table 2 (continued)

	Amplitude	R^2	Mesor	Acrophase	n
TBG					
A	7.8 ± 1.4	42 ± 6%	18.0 ± 0.9	13:00 ± 1:15	11
B	9.3 ± 1.1	46 ± 6%	18.2 ± 1.1	14:47 ± 2:28	9
n	9	9	9	9	
p-value	0.419	0.623	0.829	0.12	
T4					
A	11.4 ± 1.0	64 ± 5%	110.7 ± 4.4	12:55 ± 0:28	10
B	7.4 ± 0.7	58 ± 5%	120.6 ± 6.0	11:47 ± 1:40	11
n	10	10	10	10	
p-value	**0.016**	0.373	0.051	**0.003**	

levothyroxine and 39.5 ± 12.2 points during the bedtime administration. The RAND-36 questionnaire was also analysed and among the 8 subscales, only the subscale bodily pain changed significantly in favour of the bedtime administration of levothyroxine (p=0.016).

DISCUSSION

This study shows that thyroid hormone profiles improve strikingly after changing the time of ingestion of L-thyroxine from early in the morning, before breakfast, to late in the evening, at bedtime. This has important consequences for the millions of patients who take L-thyroxine daily. Taking L-thyroxine at bedtime is also more practical for most patients, as it does not coincide with meal times. Switching to bedtime administration also proves to be safe and was well-tolerated.

We carried out this pilot study to make sure that the time of L-thyroxine administration does not change the circadian rhythm of the serum TSH and iodothyronine levels that could result in a systematic error if a large, randomized and double-blind study of the effects of the time of L-thyroxine ingestion was to be analyzed using a single time of blood collection. The marked improvement in T4 availability after changing the time of L-thyroxine ingestion from before breakfast to bedtime which is

strongly suggested by this pilot study indeed needs to be confimed in a larger randomized trial.

Although the study design seems to be unbalanced as 2 cases took T4 in the evening first and then switched to the morning dose, the results of the study were similar in both these 2 cases and in the 10 other cases and therefore confirm the overall study outcome.

There may be several explanations for our results. Firstly, breakfast may interfere with intestinal absorption of L-thyroxine, even if eaten half an hour after ingestion of the tablet. If patients take the tablet just before bedtime, it is usually hours since their last meal. Secondly, bowel motility is slower at night (16), resulting in a more prolonged exposure of the L-thyroxine tablet to the intestinal wall and, consequently, in a better uptake. The mechanism of transport of L-thyroxine across the intestinal wall is unclear. Thus, it is not known whether this is an active or a passive process, and what factors influence this process. It is possible that this transport process plays a role in the difference in L-thyroxine uptake at different times of the day (17, 18).

Thirdly, the production and activity of deiodinases (type 1, 2 and 3) is influenced by hyper- and hypothyroidism and certain drugs (19, 20), but also a circadian rhythm of the deiodinases has recently been demonstrated. Type 2 deiodinase for example has a circadian rhythm in the central nervous system (21). Other inactivating pathways of T4 metabolism, such as glucuronidation and sulfation in liver and other tissues, may also vary during the day, and contribute to the greater bioavailability of thyroxine taken at night. However, one would expect changes in the serum T3/rT3 ratio and T4 patterns, if the metabolism of thyroid hormones is affected by changing the time of intake of levothyroxine, which was not observed.

The bioactivity of TSH also has a circadian variation, with less bioactive and differently glycosylated TSH molecules secreted during the night (22). This has been used as an explanation for why thyroid hormones do not have a nocturnal rise after the TSH surge. Whether the bioactivity of TSH is influenced by changing the time of levothyroxine ingestion is unknown.

Although we did not find a correlation in the circadian rhythms of TSH and FT4, we found – as expected - a negative correlation of the 24-h mean TSH levels with the 24-h mean FT4 levels.

In our study the magnitude and time of the nocturnal TSH surge were the same during the morning and bedtime administration of L-thyroxine. Although FT4 and T4 showed a different timing in acrophase, this was not enough to influence the timing of the TSH acrophase.

The important practical consequence of our findings, namely that the circadian rhythm of TSH does not change when switching the time of levothyroxine ingestion to bedtime, is that blood sampling for monitoring thyroid hormone replacement in patients taking L-thyroxine at bedtime need not be changed, i.e. can take place in the morning as is usually done.

The circadian rhythm of the binding proteins TBG, total protein and albumin is explained by postural changes (with lower levels in supine position). Interestingly, we also found a circadian rhythm in FT4, T4 and rT3. In previous studies only rapid fluctuations of FT4 were observed, without evidence of a circadian rhythm (23).

In conclusion, our study shows that taking L-thyroxine at bedtime significantly improves thyroid hormone profiles in patients with primary hypothyroidism, whereas the circadian rhythm of TSH remains intact. A large double-blinded randomized study will need to be performed to confirm our results.

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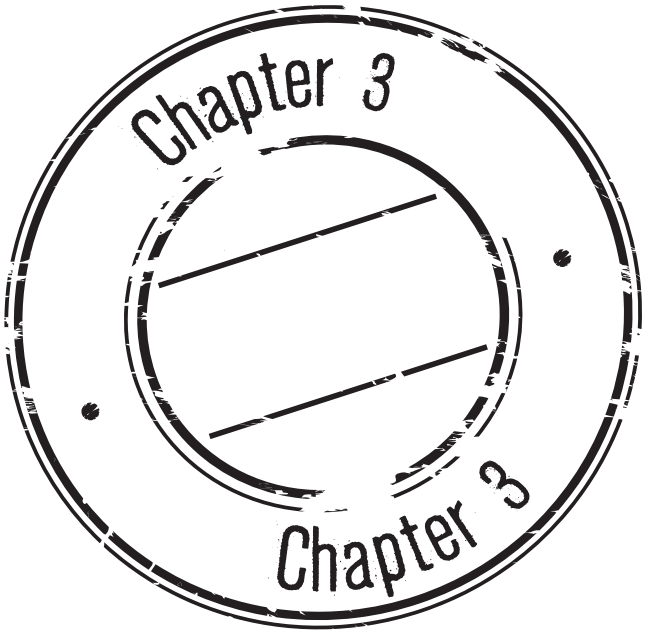

Effects of evening versus morning thyroxine intake: a randomized double-blind crossover trial

Nienke Bolk
Theo J Visser
Judy Nijman
Ineke J Jongste
Jan GP Tijssen
Arie Berghout

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ABSTRACT

Background Levothyroxine sodium is widely prescribed to treat primary hypothyroidism. There is consensus that levothyroxine should be taken in the morning on an empty stomach. A pilot study showed that levothyroxine intake at bedtime significantly decreased thyrotropin levels and increased free thyroxine and total triiodothyronine levels. To date, no large randomized trial investigating the best time of levothyroxine intake, including quality of life evaluation has been performed.

Methods To ascertain if levothyroxine intake at bedtime instead of in the morning improves thyroid hormones levels, a randomized, double-blind, crossover trial was performed between April 1, 2007 and November 30, 2008, among 105 consecutive patients with primary hypothyroidism at Maasstad Hospital Rotterdam in the Netherlands. Patients were instructed during 6 months to take 1 capsule in the morning and 1 capsule at bedtime (one containing levothyroxine and the other a placebo), with a switch after 3 months. Primary outcomes were thyroid hormone levels; secondary outcome measures were creatinine and lipid levels, body mass index, blood pressure, heart rate and quality of life.

Results Ninety patients completed the trial and were available for analysis. Compared with morning intake, direct treatment effects when levothyroxine was taken at bedtime were a decrease in TSH level of 1.25 mU/L (95% confidence interval (CI) 0.6-1.89 mIU/L, $p<0.001$), an increase in FT4 by 0.07 ng/dl (0.02–0.13 ng/dL, $p=0.01$) and an increase in TT3 by 6.5 ng/dl (0.9-12.1 ng/dL, $p=0.02$). Secondary outcomes, including the QOL-questionnaires (36-item Short Form Health Survey, Hospital Anxiety and Depression Scale, 20-item Multidimensional Fatigue Inventory and a symptoms-questionnaire), showed no significant changes between the morning vs. bedtime intake of levothyroxine.

Conclusions Levothyroxine taken at bedtime significantly improved thyroid hormone levels. Quality of life variables and plasma lipid levels showed no significant changes with bedtime vs morning intake. Clinicians should consider prescribing levothyroxine intake at bedtime.

Trial Registration: isrctn.org, Identifier: ISRCTN17436693 (NTR959).

INTRODUCTION

Because the prevalence of primary hypothyroidism is high in the general population (1,2), levothyroxine is one of the most prescribed medications. The absorption of levothyroxine takes place in the small bowel and is approximately 70-80% (3). There is consensus that levothyroxine should be taken before breakfast to prevent interference of its intestinal uptake by food or other medication (4-10). In our clinics we observed several patients whose thyroid hormone levels improved markedly after changing the scheduled intake of levothyroxine to bedtime. A pilot confirmed this observation among 11 patients (11). The mean (SD) plasma thyrotropin (TSH) level significantly decreased from 5.1 (0.9) to 1.2 (0.3) mIU/l and free thyroxine (FT4) and triiodothyronine (T3) levels increased when levothyroxine was taken at bedtime. The circadian pattern of the TSH rhythm remained intact, which was important regarding the time of blood sampling for TSH levels to monitor levothyroxine therapy.

Accordingly, we conducted a randomized double-blind crossover trial to confirm whether levothyroxine taken at bedtime leads to lower TSH and higher FT4 and T3 levels. Hypothyroidism can have major effects on health and quality of life (QOL), as it is associated with fatigue, weight gain, cold intolerance, depression, neuromuscular symptoms, diastolic dysfunction and impairment of renal function (12-15). It is also associated with risk factors for cardiovascular disease, such as hyperlipidemia, hyperhomocystinemia and arterial hypertension, notably in case of insufficient thyroid hormone supplementation (16,17).

The primary objective of this study was to determine whether a change occurred in TSH and thyroid hormone levels when levothyroxine was taken at bedtime vs in the morning. We further investigated whether a bedtime regimen would affect creatinine and lipid levels, body mass index (BMI), heart rate and QOL.

METHODS

Study participants

A randomized double-blind crossover trial was performed in consecutive patients with primary hypothyroidism that visited our clinics. The patients had to be older than 18 years of age and be on a stable dose of levothyroxine for at least 6 months. Patients with a gastrointestinal disorder, with thyroid carcinoma and pregnant women were excluded. Also patients who used intercurrent medication known to interfere with the uptake of levothyroxine were excluded (4,7-10). The Medical Ethics Committee of the Maasstad Hospital Rotterdam approved the study protocol and written informed consent was obtained from each patient.

Randomization and treatment

After informed consent had been obtained patients were randomized to start the study period with either levothyroxine in the morning (and placebo at bedtime) or levothyroxine at bedtime (and placebo in the morning). After 3 months patients were switched from levothyroxine in the morning to placebo and vice versa for another 3 months. Double blind study medication was provided by the hospital pharmacy, which performed the actual randomization. Commercial Thyrax Duotab tablets 0,100 mg were used and reformulated as capsules with lactose 1H2O as the single excipient (in compliance with GMP guidelines, annex 13, Manufacture of investigational medicinal products). Every patient got capsules containing a similar dose of levothyroxine as before entry into the trial. Placebo and levothyroxine capsules were optically identical. Patients were instructed by a research nurse to take the morning capsule on an empty stomach half an hour before breakfast and the bedtime capsule at night just before going to bed.

Data collection, follow up and compliance

At baseline and every 6 weeks people were seen in our clinics by a research nurse. At these visits blood samples were taken to determine plasma TSH, FT4, T3, creatinine and lipids and blood pressure, heart rate and body weight were measured. Also the remaining capsules in the containers were counted to check for compliance. Quality of life questionnaires were completed by the patients at baseline, at 3 months and at the end of the study.

Blood sampling

Blood samples were drawn in the morning before the planned visit to the research nurse. Capsules were not withheld on the day of bloodsampling. Serum TSH levels (reference range 0.4-4.0 mU/l) were measured by immunometric assay (Immulite 2000, DPC Nederland, Breda, The Netherlands), with a detection limit of 0.002 mU/l. FT4 (reference range 10-24.0 pmol/l, equivalent with 0.78-1.86 ng/dl) and T3 (reference range 1.23-2.80 nmol/l, equivalent with approximately 80-182 ng/dl) were measured by competitive immunoassay (Immulite 2000). Creatinine and lipid levels were measured on a Dimension RxL machine (Dade-Behring).

Quality of life and Patient preference

Three different quality of life (QOL) questionnaires (SF-36, HAD, MFI-20) and a specific questionnaire for symptoms of hypo- and hyperthyroidism were completed by the patients at baseline, at 3 months and at the end of the study. Patients completed the questionnaires under the supervision of a research nurse. The SF-36/RAND-36 (the 36-Item Short Form Health Survey questionnaire) is a general quality of life questionnaire that comprises 8 scales (18). The Hospital Anxiety and Depression Scale (HADS) consists of 14 items pertaining to anxiety and depression (19). The 20-Item Multidimensional Fatigue Index (MFI-20) measures 5 different dimensions of fatigue (20). The specific questionnaire for thyroid symptoms assesses 14 symptoms of hypothyroidism and 6 symptoms of hyperthyroidism (21). At the end of the trial, before the randomization code was broken, patients were asked during which period of the trial they had felt better. After the trial had ended patients were free to choose at what time they wanted to continue taking levothyroxine, in the morning or at bedtime. One year after the trial we asked every patient at what time they took the levothyroxine tablet.

Statistical analysis

The primary end point was a change in thyroid hormone parameters (TSH, FT4, TT3) between 12 weeks of morning thyroxine and 12 weeks of bedtime levothyroxine. Secondary end points were changes in the quality of life (measured by 3 questionnaires) and in the thyroid symptom score, body mass index (BMI), heart rate and serum lipid and creatinine levels.

The direct treatment effect in all parameters was measured by performing an independent samples t-test between the differences of week 12 and week 24 in the first group (started with morning thyroxine) and the second group (started with bedtime thyroxine) (22). The presence of a carryover effect, from one period to the other, was measured by performing a independent samples t-test on the sum of the parameters at 12 and 24 weeks in each individual. All p-values were two-sided and have not been adjusted for multiple testing. All calculations were done using SPSS 17.0 for Windows.

To calculate the sample size we assumed that a difference in TSH of 1.0 mU/l between morning and bedtime ingestion of levothyroxine would be clinically relevant. From our previous results in the pilot study we calculated that the standard deviation of the difference between morning and bedtime administration would be between 2.5 and 3.0 mU/l. Based on these calculations, a sample size of 75 patients would give at least 80% power to detect a significant difference of 1 mU/l between the morning and bedtime administration.

RESULTS

Study participants and treatment

From April 2007 through June 2008, a total of 141 patients were assessed for eligibility. Eventually 105 patients met the inclusion criteria and gave written informed consent. Reasons for exclusion were the intercurrent use of interfering medication (n=11), a history of thyroid carcinoma (n=3), an unusual dose of levothyroxine (n=6), treatment for breast cancer (n=1) and Addison's disease (n=1). Of the randomized patients 15 withdrew their consent shortly after entry into the trial and have only results at baseline. The baseline characteristics of these patients did not differ from the patients who completed the trial. Data for analysis of the 24 weeks were available for 90 (86%) of the patients who enrolled the trial, of whom 47 started with levothyroxine intake in the morning and placebo at bedtime, and 43 started with levothyroxine at bedtime and placebo in the morning (Figure 1).

The baseline characteristics of the two groups are given in Table 1. There was a difference in the amount of male patients, dose of

levothyroxine and TSH between the two groups. On average, patients missed 1.3 ± 6 capsules in the morning and 1.9 ± 10.1 capsules in the evening during the 24 weeks of the trial (p= 0.54). As there were no severe symptoms related to hypo- or hyperthyroidism, none of the patients required an adaptation of the levothyroxine dose during the trial.

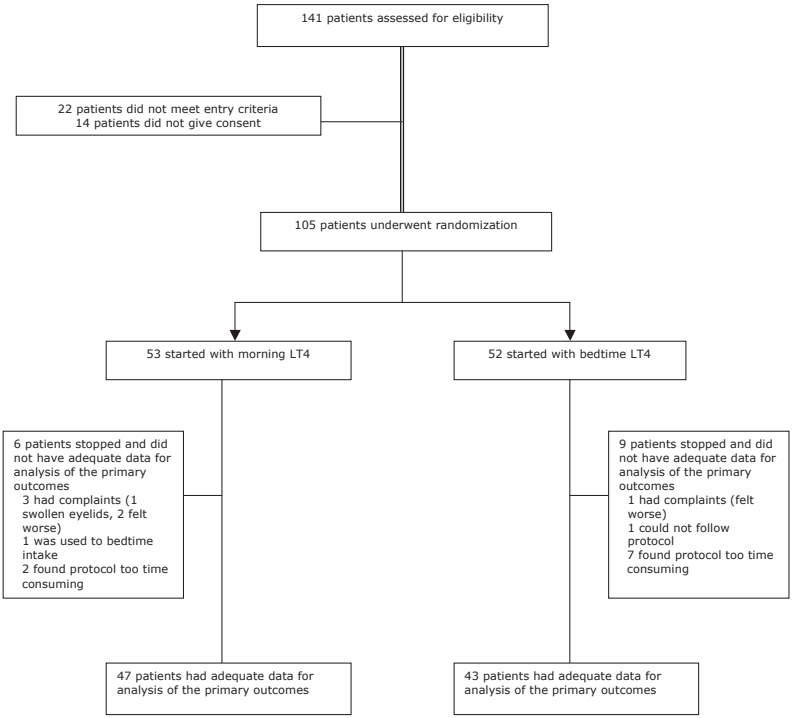

Figure 1. Flowchart of the study, showing disposition of patients from screening to study completion Consecutive patients with primary hypothyroidism that visited our clinics were assessed for eligibility. Exclusion criteria were a gastrointestinal disorder, thyroid carcinoma and pregnancy. LT4 denotes thyroxine.

Table 1 Baseline characteristics of the patients with adequate data for analysis of the primary outcomes

SI conversion factors: To convert TSH level to micrograms per liter, multiply by 1.0; free thyroxine (FT4) level to picomoles per liter, multiply by 12.871; total T3 level to nanomoles per liter, multiply by 0.0154. *The body-mass index is the body weight in kilograms divided by the square of the height in meters.

	Morning LT4 first (n=47)	**Bedtime LT4 first (n=43)**
Age (yr)	48 (11)	48 (13)
Male sex (no. of patients)	13	7
Body mass index*	29.1 (20.0-40.1)	27.9 (19.8-41.0)
Etiology of hypothyroidism (no.of patients)		
Hashimoto's	26	26
I131	17	17
Thyroidectomy	3	0
Radiotherapy neck	1	0
Duration hypothyroidism (yrs)	3 (0.5-25)	2 (0.5-21)
Dose thyroxine (mcg)	125 (50-200)	100 (50-250)
TSH (mU/l)	1.5 (1.7)	3.3 (3.0)
FT4 (ng/dl)	1.58 (0.26)	1.50 (0.27)
T3 (ng/dl)	132.9 (30.7)	122.0 (38.1)
Patients that used other medication (no. of patients)	26	25

Primary outcomes

The results of the primary outcomes are shown in Table 2 and Figure 2. In the group that received morning levothyroxine first, mean TSH (SD) dropped from 2.66 (2.53) mU/l at the end of the morning intake period to 1.74 (2.43) mU/l at the end of the bedtime intake period. In contrast, in the group that received bedtime levothyroxine first, TSH increased from 2.36 (2.55) mU/l at the end of the bedtime intake period to 3.86 (4.02) mU/l at the end of the morning intake period. When the overall changes were compared between the two groups, bedtime levothyroxine intake was found to have a direct treatment effect with a decrease

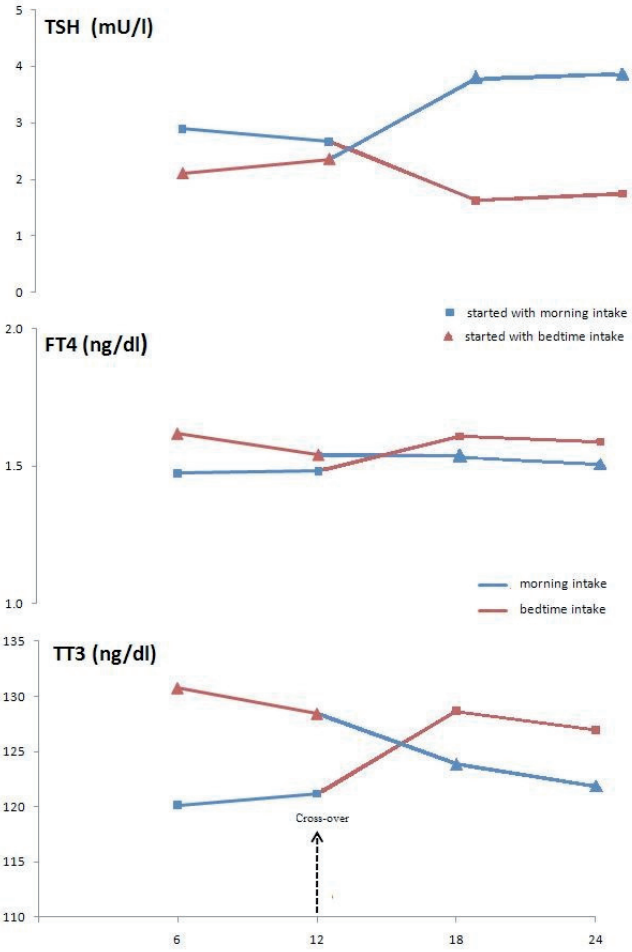

Figure 2. Thyroid hormone levels after 6 and 12 weeks of morning or bedtime intake of thyroxine

Mean TSH, FT4 and TT3 levels after 6 and 12 weeks of morning (blue) or bedtime (red) intake of thyroxine in the two randomized groups (started with morning (■) vs bedtime (▲) thyroxine). The normal range for TSH is 0.4 to 4.0 mU per liter. The normal range for FT4 is 0.78 to 1.86 ng per deciliter. The normal range of T3 is 80-182 ng per deciliter.

Table 2 Mean laboratory results and measurements after 12 and 24 weeks of morning or bedtime intake of thyroxine in the two randomized groups

SI conversion factors: To convert TSH level to micrograms per liter, multiply by 1.0; free thyroxine (FT4) level to picomoles per liter, multiply by 12.871; total T3 level to nanomoles per liter, multiply by 0.0154. creatinine level to micromoles per liter, multiply by 88.4; cholesterol level to milimoles per liter, multiply by 0.0259.

	Morning intake first (n=47)			Bedtime intake first (n=43)			Difference		Direct treatment effect	
	baseline	12 weeks	24 weeks	baseline	12 weeks	24 weeks	morning first	bedtime first	(95% confidence interval)	p-value
TSH (mU/l)	1.52 (1.71)	2.66 (2.53)	1.74 (2.43)	3.31 (3.01)	2.36 (2.55)	3.86 (4.02)	-0.92 (2.08)	1.57 (3.87)	1.25 (0.6 ; 1.89)	<0.001
FT4 (ng/dl)	1.58 (0.26)	1.48 (0.24)	1.59 (0.33)	1.50 (0.27)	1.54 (0.28)	1.51 (0.20)	0.11 (0.27)	-0.04 (0.25)	-0.07 (-0.13 ; -0.018)	0.010
TT3 (ng/dl)	132.9 (30.7)	121.2 (28.1)	127.0 (31.6)	122.0 (38.1)	128.4 (30.6)	121.9 (36.3)	5.80 (26.2)	-7.13 (26.3)	-6.46 (-12.1; -0.9)	0.024
Creatinin (mg/dl)	0.76 (0.17)	0.77 (0.21)	0.74 (0,16)	0.75 (0.28)	0.74 (0.26)	0.74 (0.29)	-0.03 (0,10)	0.00 (0,10)	0.02 (-0.001 ; 0.037)	0.127
Total cholesterol (mg/dl)	194.3 (38.5)	197.1 (38.1)	191.3 (38.3)	201.2 (44.7)	198.9 (46.6)	199.0 (43.2)	-5.75 (26.7)	0.57 (19.7)	3.16 (-1.9 ; 8.2)	0.216
BMI (kg/m^2)	29.5 (5.4)	29.7 (5.6)	29.7 (5.6)	28.7 (4.6)	28.8 (4.6)	29.1 (4.8)	0.01 (0.8)	0.28 (0.6)	0.13 (-0.02 ; 0.29)	0.088
Heart rate (bpm)	75 (11.5)	79 (12.3)	79 (13.0)	77 (11.6)	78 (11.7)	77 (11.0)	0.93 (12.7)	-1.07 (8.9)	-1.0 (-3.4 ; 1.4)	0.399

in TSH by 1.25 mU/l (95% confidence interval, CI, 0.6-1.89 mU/l, p<0.000) relative to morning levothyroxine intake.

FT4 (SD), in the group that received morning levothyroxine first, increased from 1.48 (0.24) ng/dl at the end of the morning intake period to 1.59 (0.33) ng/dl at the end of the bedtime intake period. In the group that received bedtime levothyroxine first, FT4 decreased from 1.54 (0.28) ng/dl to 1.51 (0.2) ng/dl. Thus bedtime intake resulted in a direct treatment effect with an increase in FT4 by 0.07 ng/dl (95% CI 0.02-0.13, p=0.01) relative to morning intake.

Changes in TT3 were similar to those in FT4. In the group that received morning levothyroxine first, TT3 (SD) increased from 121.2 (28.1) ng/l to 127.0 (31.6) ng/dl. And in the group that received bedtime levothyroxine first, TT3 dropped from 128.4 (30.6) ng/dl to 121.9 (36.3) ng/dl. Here the direct treatment effect of bedtime levothyroxine was an increase in TT3 by 6.5 ng/dl (95% CI 0.9-12.1, p=0.024). No first-order carry over effect was found for TSH, FT4 or TT3.

Secondary outcomes

There were no differences between serum creatinine and lipid levels, blood pressure, heart rate and BMI between the two regimens (Table 2).

The SF-36 questionnaire, the HAD- scale and the MFI-20 did not show differences in their subscales or total scores between the period of morning versus bedtime intake of levothyroxine (Table 3). Furthermore, the symptoms indicative of hypothyroidism showed no change between the two periods, despite the improved thyroid hormone profiles. Neither was there a difference in symptoms of hyperthyroidism (Table 3).

Patient preference

When asked at the end of the trial (before the randomization code was broken), 34 of the 90 patients preferred the period in which the morning capsule contained levothyroxine, 31 patients the period in which the bedtime capsule contained levothyroxine; 25 persons could not indicate a preference. More than half of the patients still preferred the bedtime intake of levothyroxine one year after the trial was completed.

Table 3 Quality of life and symptoms after 12 and 24 weeks of morning or bedtime intake of thyroxine in the two randomized groups

	Morning intake first			Bedtime intake first			Difference		Direct treatment effect	p-value
	baseline	12 weeks	24 weeks	baseline	12 weeks	24 weeks	morning first	bedtime first	95% confidence interval	
SF36										
Physical functioning	62,7 (20.2)	64,5 (19.6)	62,7 (20,9)	65,2 (17,9)	67,4 (16,4)	66,5 (16,4)	-2,56 (13,43)	-0,83 (10,93)	0,86 (-1.26; 3.50)	0,52
Social functioning	72,3 (23.6)	71,5 (26.8)	70,9 (23,0)	65,8 (27,5)	73,0 (20,6)	71,2 (24,2)	-2,22 (23,43)	-1,74 (20,52)	0,24 (-4.44; 4.92)	0,92
Role limitations due to physical problems	55,9 (42.4)	63,0 (40.7)	59,2 (41,3)	65,6 (39,9)	57,9 (42,0)	67,9 (39,5)	-4,44 (38,54)	8,75 (35,15)	6,60 (-1.40; 14.60)	0,10
Role limitations due to emotional problems	72,3 (41.3)	73,9 (37.1)	69,6 (39,0)	79,4 (35,3)	71,4 (36,5)	69,8 (40,4)	-3,70 (43,36)	-0,79 (39,98)	1,46 (-7.46; 10.37)	0,75
Mental health	70,3 (17.8)	69,6 (18.6)	68,3 (17,8)	65,2 (20,1)	63,7 (21,8)	66,0 (21,7)	-1,28 (12,82)	2,67 (13,73)	1,97 (-0.83; 4.77)	0,16
Vitality	50,0 (23.7)	55,1 (21.2)	52,0 (22,9)	51,1 (20,0)	52,2 (20,2)	52,6 (20,4)	-3,09 (20,07)	0,35 (15,75)	1,72 (-2.09; 5.52)	0,37
Pain	73,3 (26.2)	73,8 (25.3)	72,8 (23,5)	75,6 (26,7)	75,6 (28,7)	76,7 (28,0)	-1,00 (19,09)	1,09 (20,19)	1,05 (-3.07; 5.16)	0,62
General health	53,8 (22.4)	56,5 (22.7)	56,6 (21,6)	56,7 (21,9)	59,3 (23,2)	58,8 (22,7)	-1,56 (15,48)	-0,73 (13,99)	0,41 (-2.77; 3.59)	0,80
HADS										
Anxiety	6,3 (4,5)	6,5 (4,5)	5,9 (4,2)	7,0 (4,2)	7,1 (5,0)	7,2 (4,3)	-0,60 (2,53)	0,09 (2,93)	0,34 (-0.23; 0.92)	0,23
Depression	5,0 (3,8)	4,8 (4,1)	4,4 (3,8)	5,8 (4,0)	5,0 (4,5)	5,6 (5,0)	-0,45 (2,66)	0,55 (3,03)	0,50 (-0.10; 1.10)	0,10
MFI										
General fatigue	12,7 (5,3)	11,9 (4,9)	12,2 (5,0)	12,4 (5,1)	12,3 (5,1)	12,4 (5,5)	0,30 (4,13)	0,17 (3,77)	-0,07 (-0.91; 0.77)	0,88
Physical fatigue	12,5 (5,4)	11,1 (4,9)	11,7 (4,8)	12,8 (4,5)	11,8 (4,7)	11,3 (4,6)	0,55 (3,89)	-0,51 (3,82)	-0,53 (-1.34; 0.28)	0,19
Reduced activity	10,8 (4,3)	10,5 (4,1)	10,7 (4,4)	11,1 (4,5)	11,2 (4,5)	11,2 (4,1)	0,23 (3,44)	-0,10 (3,94)	-0,16 (-0.94; 0.62)	0,67
Reduced motivation	9,6 (4,3)	9,7 (4,7)	9,9 (4,5)	10,8 (4,0)	10,7 (4,4)	10,7 (4,1)	0,15 (3,75)	-0,05 (4,34)	-0,10 (-0.95; 0.75)	0,82
Mental fatigue	9,8 (5,0)	10,4 (5,1)	10,0 (4,8)	11,0 (5,4)	11,0 (5,1)	10,4 (4,2)	-0,34 (4,14)	-0,56 (3,91)	-0,11 (-0.96 ; 0.74)	0,80

Table 3 (continued)

	Morning intake first			Bedtime intake first			Difference		Direct treatment effect	p-value
	baseline	12 weeks	24 weeks	baseline	12 weeks	24 weeks	morning first	bedtime first	95% confidence interval	
Symptoms										
Hypothyroidism	29,8 (8,6)	27,2 (7,7)	28,7 (8,3)	31,5 (9,4)	29,2(8,5)	29,6(8,8)	1,45 (7,14)	0,42 (5,11)	-0,51 (-1.83; 0.80)	0,44
Hyperthyroidism	11,5 (3,7)	11,0 (3,3)	10,8 (3,5)	12,5 (3,9)	12,1(3,8)	12,0(4,3)	-0,15 (3,16)	-0,12 (2,80)	0,02 (-0.61; 0.65)	0,96

COMMENT

We performed this large randomized double-blind trial in 90 patients to address the question if levothyroxine taken at bedtime instead of in the morning improves thyroid hormone levels. The primary outcomes show a decrease in TSH level by 1.25 mU/l and increased FT4 and TT3 levels when levothyroxine is taken at bedtime. Despite the change in thyroid hormone levels, the quality of life did not differ. The intake of the levothyroxine tablet at bedtime instead of in the morning could be more convenient for patients, as they do not have to postpone breakfast. After our study was completed more than half of the patients decided to continue with the bedtime intake of levothyroxine.

How can the effects on the bioavailability of levothyroxine be explained? An interval of 30 minutes between taking levothyroxine and breakfast may be too short to prevent interference with the gastrointestinal absorption of levothyroxine. Moreover, many patients drink coffee in the morning, often instead of breakfast (6), or may take other medication which interferes with levothyroxine absorption. On the contrary, most patients in the study stated that they had taken no food or snacks for several hours before bedtime, this being their usual routine. Bowel motility is slower at night, resulting in a more prolonged exposure of levothyroxine to the intestinal wall and, consequently in better availability (23). Furthermore, basal gastric acid secretion is highest in the late evening and lowest in the morning (24). An acidic environment promotes the absorption of levothyroxine (25). These circadian differences in gastro-intestinal function could be a pathophysiological explanation for our findings.

Thyroid hormone level changes did not translate into a change in the quality of life. There are various explanations for this observation. Patients with hypothyroidism taking adequate doses of levothyroxine (i.e. those whose TSH level is in the reference range) can still have significant impairment in psychological well being and cognitive function compared with control subjects (26, 27). This could be related to their knowing that they have a chronic illness or are overweight. Weight gain and inability to lose weight are known to occur in patients with treated hypo- and hyperthyroidism (28, 29). In our study, BMI was high in both study groups. A trial investigating T3 supplementation

showed that improved QOL was limited to a subgroup of patients with suppressed TSH levels who had lost weight (30). Based on the results of population studies (2, 28) it has been suggested that the treatment goal in patients with hypothyroidism should be a TSH level of 1.0 mU/l or lower. On the other hand, plasma thyroid hormone levels may not be representative of thyroid hormone levels at the tissue level (e.g. the brain); therefore, they would be unrelated to QOL (31,32). Finally, newly discovered thyroid hormone metabolites like thyronamine (T1), that are not replaced by levothyroxine could influence QOL (33); and the presence of an autoimmune disorder (Hashimoto's thyroiditis), may have an effect on the brain and QOL, independent from thyroid hormone supplementation (34).

The primary outcomes of this study are consistent with the results of a pilot study we performed earlier (11). Two other studies on the timing of levothyroxine have been published. In a retrospective chart review in 15 nursing home residents, Elliott showed a nonsignificant decrease in TSH levels when levothyroxine intake was switched from after breakfast to midnight (35). The results of that non-randomized trial confirm the results of our study. A 3-period cross-over design (36) study showed higher TSH values when levothyroxine was taken at bedtime instead of before breakfast, but no change in FT4 and TT3 as observed in our study. The exclusion criteria for that study were extensive, and of the eligible patients only 19% were interested in participation. The study also included patients with thyroid cancer whose TSH levels were maintained at lower levels than the rest of the population. Therefore, we think that the generalizability of our study for the treatment of primary hypothyroidism is better. The lower TSH levels with levothyroxine intake before breakfast compared to bedtime intake in that study could be explained by the larger interval between levothyroxine intake and breakfast (60 min instead of 30 min). Also, in our study most patients stated that they had nothing (or only a small snack) to eat several hours before bedtime, which differs across cultures. In all the studies performed on the timing of levothyroxine, intake on an empty stomach seems to result in the best absorption of levothyroxine. Our study shows that if this 'fasting' regimen can be achieved at bedtime, thyroid hormone levels are better than with levothyroxine intake 30 minutes before breakfast.

The study design has potential limitations including order and sequence effects. We did not find a first-order carry over effect between the two periods, but did not look at other order or sequence effects. It should also be noted that this was a single-site study in the Netherlands, where eating habits might be different from habits in other countries or cultures.

Based on the results of our study, clinicians should inform hypothyroid patients that levothyroxine intake at bedtime is a good alternative for levothyroxine intake in the morning, provided that levothyroxine is taken on an empty stomach. Notably in case patients do not reach normal TSH and/or FT4 levels with morning levothyroxine intake, a switch to bedtime is recommended. Recommendations on timing of levothyroxine intake and on uptake interference by food are found in few guidelines about the management of hypothyroidism (37,38). Drug information resources and guidelines need revision in this respect.

In conclusion, bedtime intake of levothyroxine in our study significantly improved thyroid hormone levels. This might be explained by better gastrointestinal availability at night or less interference by food and/or medications. For many patients, as shown in this study, the bedtime administration is more convenient. Clinicians should inform their patients about the possibility of taking levothyroxine at bedtime. A longer period of bedtime levothyroxine therapy may be required for a change in quality of life to occur.

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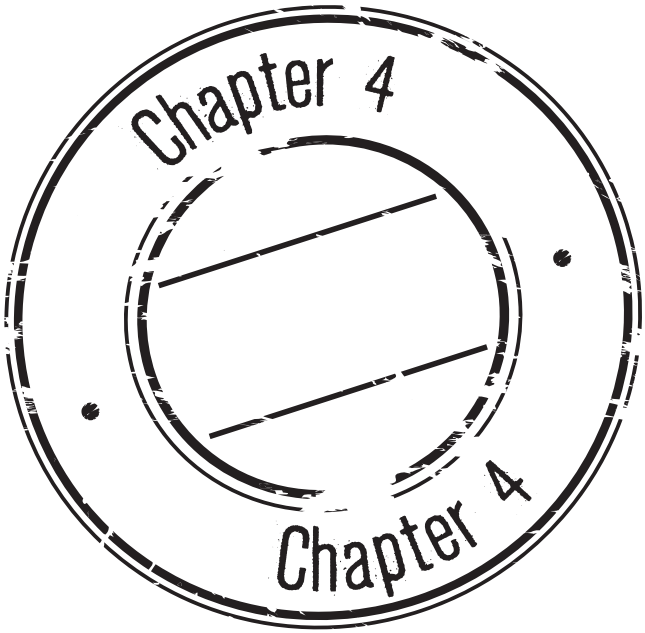

Quality of life in patients with primary hypothyroidism is related to BMI

Nienke Kelderman-Bolk
Theo J Visser
Jan P Tijssen
Arie Berghout

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ABSTRACT

Objective Many patients treated for primary hypothyroidism have an unexplained reduced quality of life (QOL). We studied the relation between QOL and various parameters in treated hypothyroid patients.

Design and Methods QOL analysis was done in 90 consecutive patients (77.8% female) treated for primary hypothyroidism. QOL was measured by the questionnaires SF-36, HADS and MFI20. Post hoc analysis was performed on the relation of QOL at baseline and BMI, thyroid hormones and other serum values. QOL in patients was also compared to the general population.

Results QOL was decreased compared to the general population. We found an inverse relationship between QOL and BMI. A relationship between QOL and serum thyroid parameters or auto-antibodies could not be found. Higher SHBG levels corresponded with a better QOL, which is explained by the negative association of SHBG with body weight and BMI.

Conclusions A decreased QOL in hypothyroid patients on thyroxine treatment is related to a higher body weight (BMI). Weight gain needs more attention in the treatment of hypothyroidism.

INTRODUCTION

Primary hypothyroidism is a frequent disorder in the general population, with a prevalence between 2% and 3% (1, 2). The symptoms of hypothyroidism vary widely and are related to a decreased function of the brain, muscles, heart and kidney (3-8). Restoration of these functions is achieved by treatment with levothyroxine (LT4) (3,5,8) , which markedly improves the signs and symptoms of hypothyroidism.

However, numerous patients treated for primary hypothyroidism with LT4, still complain of a reduced quality of life (QOL), despite serum thyroid hormone (TH) levels within the reference range (9, 10). A large study from western England showed that the QOL in a group of treated hypothyroid patients was indeed lower compared to controls of similar age and sex (9). In a study in Amsterdam in 141 hypothyroid patients, cognitive functioning, mental health and vitality were lower compared to a Dutch reference group (11).

There is no good explanation for the decreased QOL in hypothyroid patients who are euthyroid on LT4 treatment. It seems plausible that there is a correlation between QOL and thyroid parameters (TSH, FT4 and T3). Bearing this in mind, it has been attempted to improve QOL in treated patients, by e.g. aiming at a lower serum TSH level during treatment (12), by adding liothyronine (LT3) substitution to LT4 (13, 14), or by making small changes in the LT4 dose (15). Until now these attempts have not resulted in an improvement in well-being.

Another aspect in the treatment of hypothyroidism is that serum TH levels may not represent TH levels in different tissues. In a study by Escobar-Morreale in hypothyroid rats, no single dose of T4 or T3 could restore euthyroidism in all tissues at the same time (16), probably because of varying expression and function of TH transporters and deiodinases. Sex hormone binding globulin (SHBG) is known to be a marker of hepatic TH state (17) and could be a useful parameter of thyroid hormone status at tissue level.

As most patients with hypothyroidism in iodine-replete areas have auto-immune thyroiditis (18), auto-immunity per se could also be a cause of reduced QOL. A correlation between thyroid auto-antibodies and mood

and depression has already been described by some authors (19, 20). Other factors, however, that we are still unaware of, could contribute to the reduction in QOL of our hypothyroid patients.

We recently performed a double-blind randomized cross-over trial of the effects of morning versus bedtime LT4 intake (21). During the trial thyroid hormone parameters changed within the same patient, with fluctuations just within or near the reference ranges. This is comparable to the average outpatient situation, where patients come to ask for the results of the laboratory tests. Often, based on the outcomes of these test the dosage of the thyroid hormonal supplementation is adapted. QOL in our patients, however, did not change, despite the changes in thyroid hormone levels (21). During the trial thyroid parameters, markers of auto-immunity, vital parameters and QOL were assessed. This enabled us to investigate the correlation of these parameters with QOL in patients with primary hypothyroidism. In the discussion we will also review the literature on this subject.

SUBJECTS AND METHODS

Subject selection and data collection

A double-blind randomized cross-over trial over a period of 6 months, was performed in consecutive patients with treated primary hypothyroidism in our outpatient clinic, to evaluate the effect of morning versus bedtime LT4 intake. The results of this trial were published earlier (21). In brief, patients older than 18 years of age and on a stable dose of LT4 for at least 6 months were included. During the first 3 months patients took LT4 in the morning and placebo at night and switched to bedtime LT4 and morning placebo for another 3 months (or vice versa). At inclusion all patients were clinically assessed by an internist. The medical ethics committee of the Maasstad Hospital approved the study protocol, and written informed consent was obtained from each patient.

Quality of life

QOL was analysed by Short form-36 (SF-36), Hospital Anxiety and Depression Scale (HADS) and Multidimensional Fatigue Inventory (MFI-20). Data at baseline are presented here, as our previous data (21) already showed that QOL did not change during the trial with morning

versus bedtime LT4 intake. Analyses at 3 and 6 months were performed but showed the same results.

Short-form 36 (SF-36) SF-36 is a general QOL questionnaire that comprises 8 scales: physical functioning, social functioning, role limitations (physical problems), role limitations (emotional problems), mental health, vitality, pain and general health perception. Scores per scale range from 0 to 100, the highest score indicating the best state of health (22).

Hospital Anxiety and Depression Scale (HADS) The HADS consists of 14 items pertaining to anxiety and depression. Scores for the anxiety and depression subscale range from 0-21 and for the total score from 0-42. A higher score reflects more severe anxiety and depression (23).

Multidimensional Fatigue Index (MFI-20) The MFI-20 contains 20 statements to assess fatigue. From these statements 5 different dimensions of fatigue are calculated: general fatigue, physical fatigue, reduced activity, reduced motivation and mental fatigue. Scores vary between 0 and 20. Higher scores correlate with more fatigue (24).

QOL scores at baseline were compared to scores in healthy control subjects (n=440) derived from previous studies in Leiden University Medical Center (a hospital in the same region as Rotterdam) on pituitary disorders (25). In addition to this control group, we used reference data from healthy subjects of the Dutch and West-European population, obtained from studies reporting normal age-adjusted values (22, 26, 27).

Blood sampling

Serum TSH levels (reference range 0.4-4.0 mU/l) were measured by immunometric assay (Immulite 2000, DPC, Breda, The Netherlands), and FT4 (reference range 10-24 pmol/l, equivalent with 0.78-1.86 ng/dl) and T3 (reference range 1.23-2.80 nmol/l, equivalent with 80-182 ng/dl) by competitive immunoassay (Immulite 2000).

Sex hormone binding globulin (SHBG), a marker of hepatic TH status (reference range men 14.5-48.4 nmol/l, premenopausal women 26.1-110 nmol/l, postmenopausal women 14.1-68.9 nmol/l), was measured

by immunoassay (Cobas 6000, Roche Diagnostics, Almere, The Netherlands).

Anti-thyroid peroxidase (TPO) antibodies were measured by ELISA (ImmunoCAP, Phadia AB, Uppsala, Sweden; >60 IU/l considered positive) and anti-TSH receptor (TSHR) antibodies by radio-receptor assay (BRAHMS. Diagnostica GmbH, Berlin, Germany; >14 U/l considered positive). As auto-immune hypothyroidism is often associated with other autoimmune disorders, we also tested for antibodies to parietal cells (PC), present in pernicious anaemia, by ELISA and immunofluorescence (Phadia AB; >15 U/l considered positive), and antibodies to tissue transglutaminase (tTG), present in celiac disease, by ELISA (ImmunoCAP, Phadia AB; >10 U/ml considered positive).

Statistical analysis

All calculations were done using SPSS 20.0 for Windows. All p-values were two-sided. Multiple associations between QOL and various parameters were studied in retrospect, because of which correction for multiple testing was difficult and was not performed. We used independent samples t tests (equal variances not assumed) to compare patient and control data. Literature reference data used were weighted means according to the age distribution in our cohort and an unpaired t test was used to compare means. The thyroid parameters were categorized in quartiles and SHBG and BMI in tertiles before univariate ANOVA analysis. As SHBG reference values vary with sex and age (men/women, below/ above 50 years of age), patients were divided in tertiles ('low', 'normal' or 'high' SHBG) per category. To investigate the relationships of QOL with TH, SHBG and BMI, a one-way ANOVA was performed (p value for trend). Linear regression analysis was performed on the relation between QOL and BMI, unadjusted and adjusted for age, sex and TSH.

RESULTS

From April 2007 through June 2008, 90 patients with primary hypothyroidism were included in the trial and had adequate data for analysis. The mean age was 48 yr (SD 11.7), mean BMI 29.1 kg/m^2 (SD 5.1) and 77.8% was female (Table 1).

Table 1 Baseline characteristics of treated hypothyroid patients (n=90). I131 (radioactive iodide therapy), a-TPO (anti-thyroid peroxidase), a-TSHr (anti-TSHreceptor), a-PC (anti-parietal cells), a-tG (anti-transglutaminase).

Age, mean (SD), y	48 (11.7)
Female (%)	77.8
BMI, mean (SD)	29.1 (5)
Duration hypothyroidism, median (range), y	2.5 (0.5-25)
Cause hypothyroidism, No.	
Autoimmune (Hashimoto)	52
I131	34
thyroidectomy	3
neck radiation	1
Dose LT4, median (range), µg	125 (50-250)
TSH, mean (SD), mU/l	2.38 (2.55)
FT4, mean (SD), pmol/l	19.8 (3.40)
T3, mean (SD), nmol/l	1.96 (0.53)
a-TPO, no. (%)	61 (68)
a-TSHr, no. (%)	10 (11)
a-PC, no. (%)	12 (13)
a-tG, no. (%)	0 (0)

QOL in the study population

QOL at baseline was impaired on all scales, as is shown in Table 2. There was no difference in QOL with morning or bedtime intake of LT4, as was described in our previous paper (21). There was no difference in QOL according to the etiology of the hypothyroidism or duration of treatment (< 1 year, 1-5 years, >5 years) (data not shown).

QOL and thyroid hormone parameters

QOL measured by the 3 questionnaires was not dependent on serum TSH, FT4 (Table 3) and T3 (data not shown). As described in our previous paper (21), QOL scores did not change with morning or bedtime LT4 intake, despite the significant change in thyroid parameters.

Table 2 QOL in study population compared to controls and reference data.

QOL scores at baseline were compared to scores in healthy control subjects derived from previous studies in Leiden University Medical Center (a hospital in the same region as Rotterdam) on pituitary disorders (25). In addition to this control group, we used reference data from healthy subjects of the Dutch and West-European population, obtained from studies reporting normal age-adjusted values (22, 26, 27)

	study population (n=90) mean (SD)	controls (n=440) mean (SD)	P value1	Age-adjusted reference values* mean	P value2
SF-36					
•Physical functioning	64 (19)	88 (17)	<0.001	81	<0.001
•Social functioning	69 (26)	88 (19)	<0.001	87	<0.001
•Role limits physical	60 (41)	84 (31)	<0.001	79	<0.001
•Role limits emotional	76 (39)	87 (29)	0.01	85	0.03
•Mental health	68 (19)			77	<0.001
•Vitality	51 (22)			67	<0.001
•Pain	74 (26)	86 (19)	<0.001	80	0.047
HAD					
•Anxiety	6.7 (4.3)	4.1 (3.2)	<0.001	4.99	0.001
•Depression	5.4 (4.0)	2.8 (2.9)	<0.001	3.52	<0.001
Total	12 (7.6)	6.8 (5.3)	<0.001	8.41	<0.001
MFI-20					
•General fatigue	12.6 (5.2)	8.5 (4.0)	<0.001	9.9	<0.001
•Physical activity	12.6 (4.9)	7.6 (3.7)	<0.001	8.8	<0.001
•Reduced activity	11 (4.4)	7.2 (3.5)	<0.001	8.7	<0.001
•Reduced motivation	10.2 (4.2)	7.3 (3.4)	<0.001	8.2	<0.001
•Mental fatigue	10.4 (5.2)	7.8 (3.9)	<0.001	8.3	<0.001
P value1	Patients compared with controls				
P value2	Patients compared with literature reference data				
*	Derived from Refs 22, 26, 27				

QOL and BMI

BMI in the study population (BMI 29.1 kg/m^2) was higher than in the general Dutch population (mean BMI women 25.1 kg/m, mean BMI men 26.0 kg/m) (29). QOL decreased with increasing body weight and, consequently, an increasing BMI in 11 out of 15 subscales of QOL (Table 4 and Supplemental Figure 1). The SF-36 questionnaire revealed more problems with physical functioning, role limitations due to physical problems, vitality, pain and general health at a higher BMI. The HADS questionnaire showed higher scores for depression, not anxiety. The

MFI-20 questionnaire showed more fatigue at a higher BMI on all 5 different dimensions of fatigue. There was no relation between etiology of hypothyroidism or duration of treatment and BMI (data not shown).

No relationship was found between BMI and the thyroid parameters. Adjusting for the confounding factors age, sex and TSH did not change the relation between QOL and BMI as assessed by linear regression analysis (Supplemental Table 1).

Table 3 TSH (mU/l) and FT4 (pmol/l) levels at baseline in quartiles related to QOL in treated hypothyroid patients

	TSH < 0.72	0.72 <TSH < 1.5	1.5 < TSH < 3.3	TSH > 3.3	p-value for trend
	n=22	n=23	n=23	n=22	
	mean (SD)	mean (SD)	mean (SD)	mean (SD)	
SF-36 questionnaire					
Physical functioning	66 (22)	59 (18)	65 (20)	65 (17)	0.95
Social functioning	67 (24)	63 (26)	82 (22)	65 (28)	0.61
Role-physical	65 (42)	53 (42)	62 (44)	62 (39)	0.99
Role-emotional	85 (34)	68 (44)	80 (37)	70 (38)	0.37
Emotional wellbeing	70 (17)	62 (20)	77 (13)	62 (22)	0.66
Vitality	52 (19)	42 (23)	58 (25)	50 (18)	0.53
Pain	76 (30)	77 (23)	72 (27)	74 (26)	0.69
General health	55 (24)	53 (22)	58 (21)	55 (22)	0.92
HADS					
Depression	4.8 (3.7)	6.3 (4.4)	4.7 (3.3)	5.8 (4.1)	0.69
Anxiety	6.2 (41)	7.4 (5.0)	5.6 (4.2)	7.4 (3.9)	0.68
Total	11.0 (7.2)	13.7 (8.9)	10.2 (6.7)	13.0 (7.2)	0.72
MFI-20					
General fatigue	13.1 (5.5)	14.4 (4.1)	9.8 (5.5)	13.1 (4.5)	0.32
Physical fatigue	12.4 (5.1)	14.0 (4.1)	11.0 (5.7)	13.0 (4.6)	0.82
Mental fatigue	11.1 (5.4)	11.4 (4.2)	9.2 (5.4)	9.8 (5.7)	0.22
Reduced activity	10.7 (4.6)	12.2 (3.6)	10.2 (5.4)	10.8 (3.8)	0.67
Reduced motivation	9.2 (4.2)	11.7 (4.0)	9.2 (4.4)	10.4 (3.9)	0.79

Table 3 (continued)

	FT4<17.5	17.5<FT4<20.2	20.2<FT4<21.4	FT4>21.4	p-value for trend
	n=21	n=24	n=22	n=23	
	mean (SD)	mean (SD)	mean (SD)	mean (SD)	
SF-36 questionnaire					
Physical functioning	61 (19)	64 (18)	68 (22)	64 (17)	0.35
Social functioning	70 (24)	71 (28)	74 (23)	63 (28)	0.47
Role-physical	54 (43)	57 (41)	73 (42)	60 (40)	0.38
Role-emotional	81 (37)	68 (42)	89 (24)	70 (43)	0.77
Emotional wellbeing	71 (20)	67 (20)	70 (16)	66 (18)	0.50
Vitality	52 (22)	52 (19)	53 (21)	47 (25)	0.54
Pain	78 (27)	77 (24)	75 (26)	68 (29)	0.22
General health	57 (23)	49 (18)	64 (21)	53 (24)	0.80
HADS					
Depression	4.9 (4.3)	6.1 (3.7)	4.1 (2.6)	6.2 (4.5)	0.66
Anxiety	5.7 (3.9)	7.4 (4.8)	6.1 (3.9)	6.9 (4.3)	0.66
Total	10.4 (7.2)	13.5 (8.0)	10.1 (5.7)	13.1 (8.4)	0.55
MFI-20					
General fatigue	12.0 (4.6)	12.1 (4.8)	12.1 (5.5)	13.7 (5.6)	0.31
Physical fatigue	12.2 (4.8)	12.5 (4.6)	11.7 (5.4)	13.7 (5)	0.51
Mental fatigue	10.0 (5.1)	10.0 (5.3)	10.5 (5.5)	10.5 (4.9)	0.77
Reduced activity	11.2 (5.1)	10.2 (4.3)	10.4 (4.2)	12.0 (4.1)	0.59
Reduced motivation	10.7 (4.4)	9.6 (3.9)	9.3 (3.8)	11.2 (4.7)	0.72

QOL and SHBG

A higher SHBG, a marker of hepatic TH status, corresponded with a better QOL in 9 out of the 15 subscales (Table 5). An improvement in QOL was seen in physical functioning, role limitations due to emotional problems, vitality, pain and general health, according to the SF-36 questionnaire. The HADS revealed an improvement in depression scores with higher SHBG levels, and the MFI-20 showed a reduction in physical fatigue, in reduced activity and in reduced motivation. SHBG was not related to FT4 or T3 levels.

Table 4. QOL at baseline in normal weight, overweight and obese patients treated for hypothyroidism.

	Normal weight (n=21)	Overweight (n=31)	Obese (n=38)	
	BMI < 25	25 <BMI> 30	BMI> 30	p-value for trend
	mean (SD)	mean (SD)	mean (SD)	
SF-36 questionnaire				
Physical functioning	77 (13)	61 (19)	60 (16)	0.001
Social functioning	76 (27)	72 (23)	64 (26)	0.10
Role-physical	86 (31)	60 (39)	49 (43)	0.001
Role-emotional	85 (30)	77 (35)	72 (44)	0.22
Emotional wellbeing	69 (18)	70 (13)	67 (22)	0.68
Vitality	62 (19)	48 (24)	47 (20)	0.01
Pain	90 (20)	69 (26)	71 (27)	0.007
General health	68 (20)	55 (21)	50 (22)	0.003
HADS				
Depression	3.8 (3.2)	5.2 (3.0)	6.3 (4.6)	0.02
Anxiety	6.3 (4.2)	6.7 (4.0)	6.5 (4.5)	0.83
Total	10.0 (7.0)	11.9 (6.4)	12.7 (8.4)	0.21
MFI-20				
General fatigue	10.7 (4.9)	12.9 (5.3)	13.2 (4.9)	0.08
Physical fatigue	9.4 (4.9)	12.5 (4.9)	14.0 (4.3)	0.001
Mental fatigue	7.7 (4.0)	11.0 (4.5)	11.0 (5.7)	0.02
Reduced activity	8.0 (3.4)	11.2 (5.1)	12.2 (3.7)	0.001
Reduced motivation	8.0 (3.8)	10.6 (4.3)	11.0 (4.1)	0.01

An increase in SHBG corresponded with a decrease in BMI. A 'low', 'normal' and 'high' SHBG corresponded with a BMI of 31.0 kg/m², 30.1 kg/m² and 26.3 kg/m² respectively ($p<0.001$). When corrected for BMI the correlation between SHBG and QOL disappeared in the above mentioned QOL scales, except for SF-36 role limitations due to emotional problems and MFI-20 reduced motivation scale.

Table 5. SHBG at baseline in tertiles related to QOL parameters
SHBG was divided in tertiles in the different populations (men, women<50 years, women>50 years). These tertiles were categorized as 'low', 'normal' and 'high' SHBG respectively. The tertiles of SHBG were related to QOL questionnaires in treated hypothyroid patients.

	Low SHBG (n=30)	Normal SHBG (n=30)	High SHBG (n=30)	p-value for trend
	mean (SD)	mean (SD)	mean (SD)	
SF-36				
Physical functioning	58 (22)	62 (15)	71 (18)	0.02
Social functioning	68 (22)	60 (28)	78 (23)	0.11
Role-physical	58 (44)	49 (40)	73 (37)	0.16
Role-emotional	64 (44)	69 (42)	93 (19)	0.004
Emotional wellbeing	68 (19)	63 (21)	73 (16)	0.29
Vitality	45 (24)	45 (17)	60 (21)	0.008
Pain	67 (29)	69 (26)	87 (19)	0.004
General health	53 (22)	47 (19)	66 (22)	0.02
HADS				
Depression	5.9 (4.3)	6.3 (3.8)	3.9 (3.3)	0.047
Anxiety	7.1 (4.4)	7.4 (4.4)	5.5 (4.2)	0.14
Total	13.1 (8.0)	13.6 (7.3)	9.4 (6.9)	0.06
MFI-20				
General fatigue	13.0 (4.7)	13.7 (5.0)	11.3 (5.5)	0.21
Physical fatigue	13.3 (4.8)	14.1 (4.0)	10.7 (5.4)	0.04
Mental fatigue	10.1 (4.6)	11.9 (5.3)	9.3 (5.4)	0.55
Reduced activity	12.2 (4.5)	11.5 (3.6)	9.3 (4.6)	0.01
Reduced motivation	10.7 (4.3)	11.8 (4.1)	8.3 (3.5)	0.03

QOL and autoimmunity

In 61 patients (68%), TPO antibodies were found, and 10 patients (11%) had TSHR antibodies. Twelve patients had PC antibodies (positive by both immunofluorescence and ELISA) and none of the patients had tTG antibodies. No relationship was found between QOL and any of these antibodies.

QOL compared to external controls

QOL in our study population was compared to QOL in healthy controls (Leiden controls, n=440) by SF-36, HADS and MFI-20 questionnaires (Table 2). The Leiden control group had a similar sex distribution, but was 3 years older than our patient group (p=0.04). SF-36 scores in hypothyroid patients were markedly lower than in controls in all 8 subscales, indicating a worse general QOL. Scores on the HADS and MFI-20 questionnaires were higher compared to the reference values, indicating more depression and anxiety, and fatigue respectively.

A comparison of QOL scores in hypothyroid patients with age-adjusted reference values from previous studies, confirmed the reduced QOL in the study population on all the measured subscales (Table 2).

DISCUSSION

In this study, we found that in treated hypothyroid patients, QOL was not related to the serum levels of TSH, FT4 or T3. This reinforces results from other studies, in which no correlation between QOL and thyroid parameters could be found. For example, a study on the starting dose of LT4 in newly diagnosed hypothyroidism, found a faster normalization of thyroid parameters with a high starting dose of LT4 compared with an incremental dose regimen, but did not find a faster improvement of QOL (30). Two studies on the influence of small changes in LT4 doses, and thus TH levels, could not find changes in well-being, hypothyroid symptoms, or QOL (15, 31). Our findings could have consequences for clinical practice as more than often LT4 dosages are adjusted based on the suggested relationship of patient complaints about QOL and TH levels just within or outside the normal reference range.

Interestingly, a decreased QOL was related to increasing body weight and BMI. Overweight is probably an overlooked aspect in patients with hypothyroidism, and could be a large contributor to the reduced QOL. In a study on reduced QOL in treated hypothyroid patients, putting on weight was significantly more often indicated as a problem by patients than by healthy controls (9). Even after therapy is started and the excess body water that is associated with myxedema in hypothyroidism is lost (32), the weight often remains a problem. Several studies have

indeed demonstrated that body weight of patients with primary or iatrogenic hypothyroidism increases over the years (33-35). The greater weight gain described in the studied subjects compared to age- and gender-matched controls, is not due to inadequately normalized TSH values (9, 35). Possible causes of weight gain in hypothyroid patients may include a failure of LT4 to serve as a full replacement for THs. Although FT4 and T3 levels are adequately normalized by LT4 monotherapy (36), it is difficult to mimic normal hormone secretion by hormonal substitution therapy in hypothyroid patients (37). In a trial comparing combined T4/T3 treatment with LT4 monotherapy, there was a preference of patients for combination therapy if they lost weight (11). A similar patient preference for desiccated thyroid hormone over LT4 was also connected to weight loss (33).

Obesity in general is known to decrease health-related QOL (38). Interestingly, in a Dutch study on QOL in obese and non-obese subjects, QOL in obese women was decreased on the same subscales as in our population (28). Besides the impact on QOL, obesity is also related to health problems such as hypertension, diabetes, cardiovascular disease and malignancy (39). Once people have gained weight, most interventions directed at weight loss are unsuccessful. It is therefore of the utmost importance to prevent weight gain by counselling patients and encouraging them to engage in weight loss programs. The experience of endocrinologists shows, however, that it is difficult to prevent and/or treat weight gain in hypothyroid patients. Further investigation is warranted on the cause of weight gain in (treated) hypothyroidism, which will hopefully improve treatment of hypothyroid patients in the future.

SHBG is known to be a marker of hepatic TH state (17). In our study a higher SHBG corresponded with a better QOL on various subscales. However, this relationship of SHBG with QOL disappeared in most of the subscales after correction for BMI. The level of SHBG varies with nutritional status (and thus BMI), age and sex hormones which interferes with its use as a sensitive parameter of TH action (40). As the majority of hypothyroid patients are female (with fluctuations in estrogen levels during life), SHBG is not a suitable marker of TH status. In fact, TSH itself is an indicator of tissue TH levels because of the

feedback of TH on the hypothalamus-pituitary-thyroid axis. However, as mentioned before, TSH did not show a correlation with QOL.

A relationship between QOL and thyroid auto-antibodies, or antibodies associated with other auto-immune disorders was not found in our study population. Recently, some investigators found a relation between thyroid auto-antibodies and mood and depression in subjects with a normal thyroid function (19, 20). However, this is not confirmed by others (41), which is in line with our results. Perhaps the study population needs to be larger do draw definite conclusions on this matter. We found a much higher percentage of patients with PC antibodies than previously described in a population of patients with auto-immune hypothyroidism (13% vs 4.5%) (42). We, however, measured PC antibodies, irrespective of symptoms or anaemia, and not only in patients with self-reported pernicious anaemia, which is of course a smaller proportion of subjects.

In our study population, QOL was decreased compared to the general population (26). These findings confirm the reduced QOL found in previous studies of treated hypothyroid patients (9, 11). Comparing patients with chronic illnesses, QOL in hypothyroid patients is worse than in patients with diabetes mellitus (43), but better than in patients with fibromyalgia (44). It should be mentioned that QOL in our outpatient study population could be worse than QOL in hypothyroid patients in general, because of a referral bias of patients with more complaints towards an outpatient clinic. However, it is the experience of many clinicians, including general pratitioners, that QOL related problems tend to persist in treated hypothyroid patients.

In conclusion, in a large group of treated hypothyroid patients, we found a decreased QOL that was related to an increased body weight and BMI. QOL was not dependent on serum thyroid parameters or auto-antibodies. These findings are important, as an increase in body weight is a frequent finding in treated hypothyroid patients, and should have our attention in the treatment of hypothyroidism and in future research.

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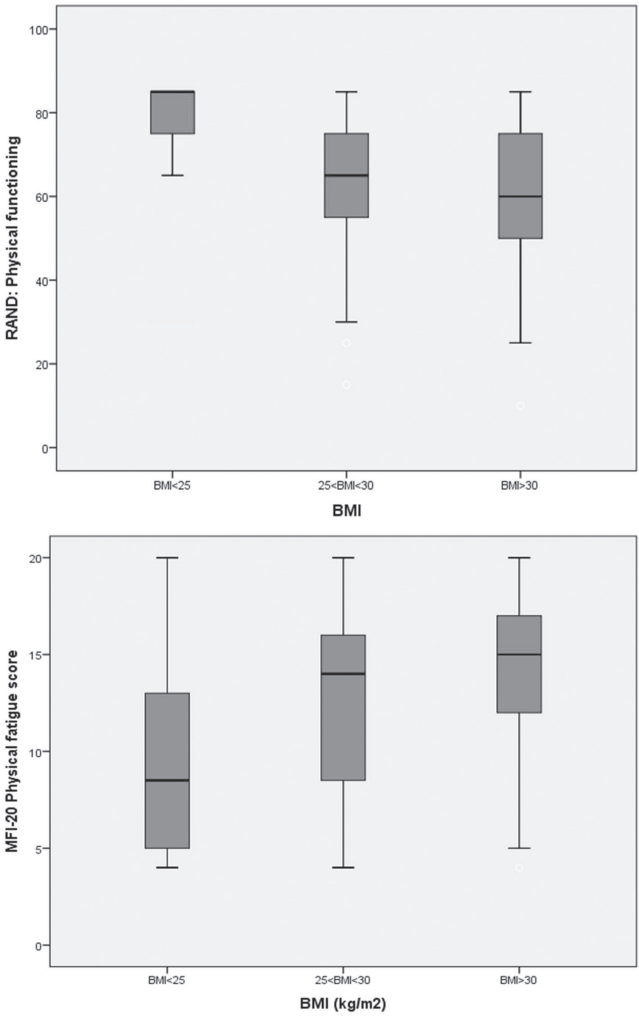
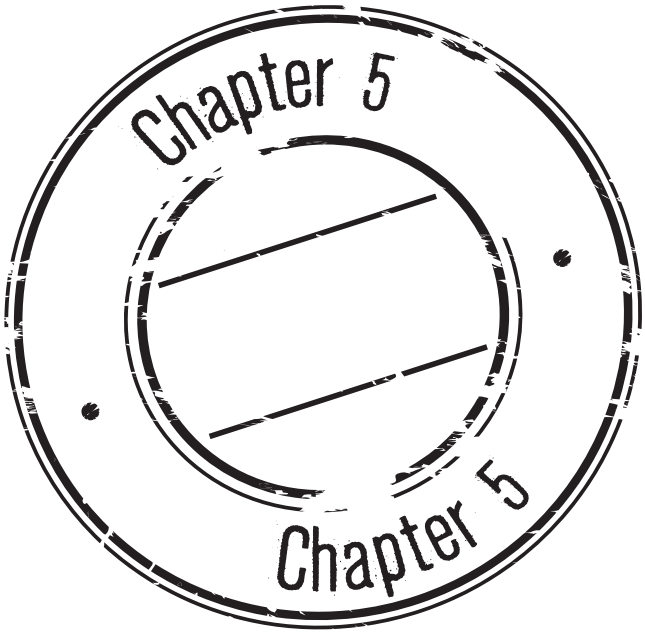


Supplemental Figure 1.
Correlation between a) BMI tertiles and SF36 (RAND) physical functioning and b) BMI and MFI20 physical fatigue. BMI (body mass index) in kg/m^2.

Supplemental Table 1 BMI at baseline (in tertiles) related to QOL unadjusted vs adjusted for age, sex and TSH (linear regression analysis)

	Coefficient (unadjusted)	P	Coefficient (adjusted for age, sex, TSH)	P
SF-36 questionnaire				
Physical functioning	-7.66	0.001	-8.09	0.001
Social functioning	-6.04	0.08	-6.06	0.08
Role-physical	-17.6	0.002	-18.1	0.001
Role-emotional	-6.33	0.22	-7.32	0.16
Emotional wellbeing	-1.32	0.6	-1.49	0.55
Vitality	-6.64	0.02	-6.65	0.02
Pain	-8.3	0.02	-8.68	0.02
General health	-8.67	0.004	-8.97	0.003
HADS				
Depression	1.25	0.02	1.36	0.01
Anxiety	0.08	0.89	0.17	0.76
Total	1.25	0.22	1.46	0.15
MFI-20				
General fatigue	1.12	0.1	1.06	0.13
Physical fatigue	2.23	0.001	2.24	0.001
Mental fatigue	1.43	0.04	1.36	0.05
Reduced activity	1.93	0.001	2.04	0.001
Reduced motivation	1.35	0.02	1.4	0.02



Transport of thyroid hormone in an intestinal cell model

Nienke Kelderman-Bolk
Chantal Zevenbergen
E.C. Lima de Souza
W. Edward Visser
Ramona E.A. van Heerebeek
Robin P. Peeters
Theo J. Visser

Submitted

ABSTRACT

Uptake of thyroid hormone (TH) by the small bowel is important for the enterohepatic cycle of TH and for absorption of TH in patients treated for hypothyroidism. TH uptake takes place in the small intestine, but the mechanism of uptake remains to be elucidated. Therefore, we characterized intestinal TH transport, using the human colorectal adenocarcinoma cell line Caco2 as a model.

T4 uptake by Caco2 was Na^+ independent, but highly dependent on pH. T4 uptake was markedly higher at pH 5.3 than at pH 7.3. At acidic pH, T4 uptake was inhibited by leucine and 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH), prototypic ligands for the L-type amino acid transporters, suggesting that T4 is transported in Caco2 cells by an L-type amino acid transporter at low pH. LAT1-transfected COS1-cells showed the same characteristics of T4 uptake as Caco2 cells and RT-qPCR analysis showed abundant mRNA expression of LAT1 in our cell line.

T3 uptake by Caco2 cells was both Na^+ and pH independent. T3 uptake was inhibited by tryptophan and verapamil but not by leucine and BCH. This suggests the involvement of a T-type amino acid transporter, most likely MCT10. RT-qPCR demonstrated abundant MCT10 mRNA expression in our Caco2 cells. Remarkably, the addition of BSP, which is a prototypic ligand for organic anion transporting polypeptides as well as for multidrug resistance-related efflux transporters, resulted in a marked increase in uptake of T3 and in particular T4.

Our data provide evidence that amino acid transporters are important for uptake of T4 and T3 by Caco2 cells. T3 uptake appears to be mediated largely by MCT10, and T4 uptake by LAT1, in particular at low pH.

INTRODUCTION

In euthyroid subjects, thyroid hormone (TH) transport across the intestinal mucosa is an important step in the enterohepatic cycle of the hormone. Also, oral replacement therapy of hypothyroid patients requires the intestinal absorption of L-thyroxine. Therefore, uptake of T3 and T4 by the intestinal wall is clearly important in maintaining stable TH levels. Previous studies have shown that the absorption of oral L-thyroxine takes place in the small bowel but the mechanisms of translocation across the mucosa are unclear (1, 2). Absorption ranges from 60% to 80% (3, 4) and is influenced by several factors, including dietary habits (5-7), interference by other drugs (8-12) and intestinal disorders (13, 14). Earlier studies have shown that the characteristics of TH uptake differs between different cell types, with regard to ligand specificity, Na^+ dependence and interactions with a variety of compounds (15). Several TH transporters have been identified in recent years, including different organic anion transporting polypeptides (OATPs) (16), Na^+/taurocholate-cotransporting polypeptide (NTCP) (17), fatty acid translocase (FAT or CD36) (18), L-type amino acid transporters LAT1 and LAT2 (19), and monocarboxylate transporter 8 (MCT8) and MCT10 (20). Multidrug resistance-associated proteins can function as an efflux transporter of TH (21).

The human colorectal adenocarcinoma cell line Caco2 is morphologically and functionally similar to human small intestinal epithelial cells making it a useful model for intestinal absorption of various compounds (22, 23). Upon continued culture for 2-3 weeks after reaching confluence, Caco2 cells differentiate spontaneously into enterocyte-like cells with increased expression of certain transporters and enzymes (22).

The aim of the present study was to characterize the transport of T4 and T3 in Caco2 cells, and thereby identify candidate transporters for TH uptake and efflux in the intestinal wall.

MATERIALS AND METHODS

Materials

[^{125}I]T_3 and [^{125}I]T_4 were prepared as previously described (24). Nonradioactive iodothyronines were obtained from Henning (Berlin, Germany). Bromosulphthalein (BSP), 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH), verapamil (VER), cyclosporin A (CSA), and the amino acids tryptophan (Trp), leucine (Leu), tyrosine (Tyr) and phenylalanine (Phe) were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands [NL]).

To obtain FLAG-LAT1-2.pcDNA3, the full-length cDNAs for human LAT1-2 were obtained from Thermo Fisher Scientific (Landsmeer, NL) and subcloned into the expression vector pcDNA3 using oligonucleotides (Integrated DNA Technologies, Leuven, Belgium) containing a FLAG tag and suitable restriction sites. All constructs were checked by sequencing (BaseClear, Leiden, NL). Mouse CD98.pcDNA3 was kindly provided by Dr. Gerd Krause (Leibniz-Institut für Molekulare Pharmakologie, Berlin, Germany).

Cell culture

Caco2 cells were obtained from the European Collection of Cell Cultures ECACC (Salisbury, UK). The cells were used between passages 42 and 60, and cultured as monolayers in 6-well culture dishes (Corning, Schiphol, NL) in DMEM/F12 medium supplemented with 9% heat-inactivated fetal bovine serum and 100 nM Na_2SeO_3 at 37 °C in a humidified atmosphere of 5% CO2-95% air. The cells were seeded at 300,000 cells/well and grown until confluence or cultured for an additional 14 days, in which case the culture medium was replaced every other day.

COS1 cells were cultured at 37 °C and 5% CO_2 in 75 cm^2 flasks with DMEM/F12 (Life Technologies, Bleiswijk, NL) supplemented with 9% heat-inactivated fetal bovine serum (Sigma Aldrich), penicillin-streptomycin, and 100 nM Na_2SeO_3. At confluence, cultured cells were split and seeded in 24-well dishes. At 70% confluence, cells were transiently transfected using X-treme Gene 9 Transfection Reagent (Roche, Almere, NL). The cells were transfected with 250 ng pcDNA3

(empty vector, EV) or with 50 ng FLAG-LAT1-2.pcDNA3 plus 50 ng CD98. pcDNA3 supplemented with 150 ng pcDNA3.

TH transport experiments

Caco2 cells were washed with incubation medium (142.9 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.8 mM $CaCl_2$, 20 mM HEPES, 0.1% glucose and 0.1% BSA, pH 7.3) or phosphate buffered saline (PBS) with 0.1% glucose and 0.1% BSA. Uptake of iodothyronines was tested by incubation of the cells for 5-180 min at 37 °C with 1 nM ($2x10^5$ cpm) [^{125}I]T_3 or [^{125}I]T_4 in 1.5 ml incubation medium. After incubation, cells were washed with medium and lysed with 1 ml 0.1 M NaOH. Radioactivity in the lysate was measured in a γ-counter. The uptake experiments were performed in the absence or presence of varying concentrations of nonradioactive iodothyronines, 1 mM BCH, Leu, Phe, Tyr or Trp, or 0.1 mM BSP, VER or CSA.

To evaluate the Na^+ dependence of T_3 or T_4 uptake, Na^+ in the incubation medium was replaced by an equimolar amount of choline. To evaluate the pH dependence of T3 or T4 uptake, the pH of the incubation medium was adjusted from 7.3 to 6.3 or 5.3.

Uptake by LAT1 and LAT2

Two days after transfection, COS1 cells were washed with assay buffer (D-PBS + Ca^{2+}/Mg^{2+} + 0.1% glucose) and incubated for 30 minutes at 37 °C with 10 nM (50,000 cpm) [^{125}I]T_4 or [^{125}I]T_3 in 0.5 ml assay buffer with or without 0.1% BSA and with or without 1 mM BCH. After incubation, cells were washed with assay buffer, lysed with 0.1 M NaOH, and counted.

RT-qPCR of TH transporters in Caco2 cells

Caco2 cells were plated in 12-well plates at $2x10^5$ cells/well and cultured for 4 or 21 days. Total RNA was isolated using the High Pure RNA Isolation kit (Roche), according to the manufacturer's instructions. One µg total RNA was reversely transcribed using the Transcriptor High Fidelity cDNA Synthesis kit (Roche). Quantitative real-time PCR was performed using the qPCR Core kit for SYBR® Green I No dUTP (Eurogentec, Maastricht, NL). Cyclophilin A (peptidylprolyl isomerase A, PPIA) was used as housekeeping gene for normalization of mRNA amplification. The primers used for qPCR are listed in Supplemental Table 1.

Statistical analysis

All results are the means ± SEM of at least duplicate determinations from representative experiments. Statistical significance was determined using the Student's t test for unpaired observations.

RESULTS

General transport characteristics

T4 and T3 uptake by Caco2 cells was measured at different incubation times. Uptake of both T4 and T3 increased over time, with T3 uptake being 3-4 fold higher than T4 uptake using medium with 0.1% BSA at pH 7.3 (Figure 1).

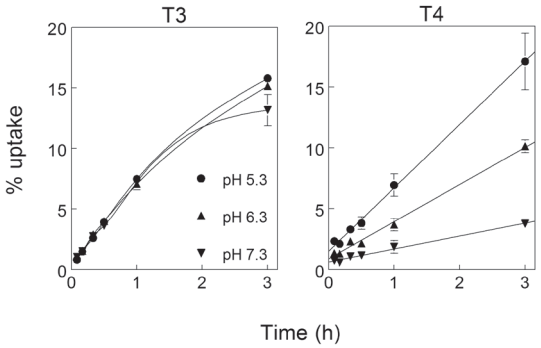


Figure 1.
Time course of uptake of 1 nM [125I]T3 and [125I]T4 by Caco2 cells at different pH values (5.3, 6.3, 7.3) in phosphate buffered saline with 0.1% glucose and 0.1% BSA Mean ± SD of duplicate measurements.

T4 transport was highly influenced by the pH of the medium, with a 3-4 fold higher uptake at pH 5.3 than at pH 7.3 (Figures 1-3), whereas T3 uptake was not significantly affected by the pH value. There was a small increase in T4 uptake at pH 5.3 by cells cultured for 21 days compared with cells cultured for 4 days. T3 uptake was slightly reduced comparing cells cultured for 21 vs. 4 days (Figure 2).

To exclude the possibility that the increased T4 uptake at a lower pH was caused by a decreased T4 binding to BSA, the experiments were

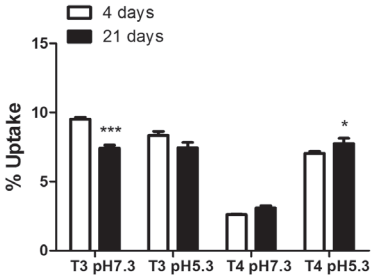


Figure 2. Uptake of T3 and T4 in different pHs in Caco-2 cells after 4 days and 21 days culture. N=2. ***p<0.001; *p<0.05 t test

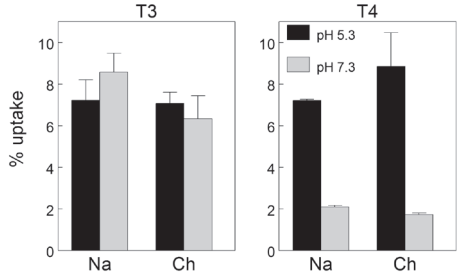


Figure 3. Na^+ and pH dependence of 1 nM [^{125}I]T4 and [^{125}I]T3 uptake by Caco2 cells during incubation for 60 min in incubation medium (142.9 mM NaCl or choline choride, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.8 mM $CaCl_2$, 20 mM HEPES, 0.1% BSA and 0.1% glucose, pH 5.3 or 7.3). Mean ± SEM, n=2.

also performed in the absence of BSA, which showed that the uptake of T4 and T3 was markedly higher compared with medium containing 0.1% BSA. The influence of pH on T4 uptake, however, was unaffected (Figure 4). To investigate the Na^+ dependence of T4 and T3 transport, we performed experiments using standard incubation medium or medium where Na^+ was replaced with equimolar amounts of choline. The results indicated that both T4 and T3 transport are Na^+ independent (Figure 3).

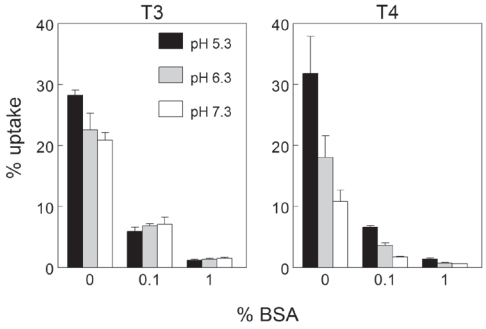


Figure 4. Effect of BSA (0, 0.1 or 1%) and pH (5.3, 6.3 or 7.3) on the uptake of 1 nM [125I]T3 and [125I]T4 uptake by Caco2 cells during incubation for 60 min in PBS with 0.1% glucose. Mean ± SEM, n=3.

Kinetic studies with increasing concentrations of substrate showed saturation of T4 transport at pH 5.3, with an apparent Km value of about 10 μM, but no saturation at pH 6.3 and 7.3 (Figure 5). Saturation of T3 transport was independent of the pH of the incubation medium, with an apparent Michaelis constant (Km) value between 1-10 μM.

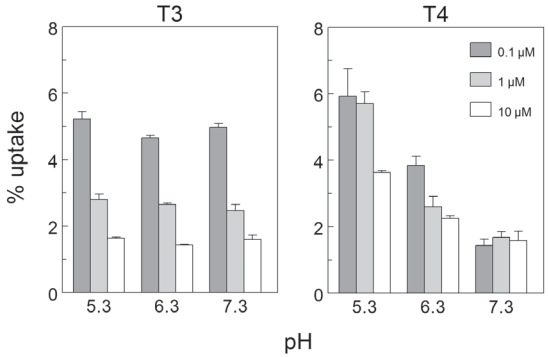


Figure 5. Effects of increasing substrate concentrations (0.1, 1, 10 μM) on the uptake of [125I]T3 and [125I]T4 during incubation for 60 min in PBS with 0.1% glucose and 0.1% BSA (pH 5.3, 6.3, 7.3). Mean ± SEM, n=5.

Inhibition of T4 and T3 transport by competitive transporter substrates

The characteristics of T4 and T3 transport in Caco2 cells were further studied by testing the effects of prototypic ligands for L- and T-type amino acid transporters, OATPs and multidrug-resistance (MDR) related efflux transporters.

At pH 7.3, uptake of T4 was inhibited by 24% with the T-type amino acid transporter substrate Trp but not by the L-amino acid transporter substrates Leu and BCH, all tested at 1 mM (Figure 6). However, at lower pH, T4 uptake became progressively inhibited by Leu and BCH up to 37% at pH 5.3. T4 uptake was also inhibited by 33% at pH 7.3 and by 27% at pH 6.3 by 0.1 mM VER, a calcium channel blocker which also inhibits P-glycoprotein (25), but it was not inhibited by 0.1 mM CSA, another P-glycoprotein inhibitor (25), irrespective of pH (Figure 7).

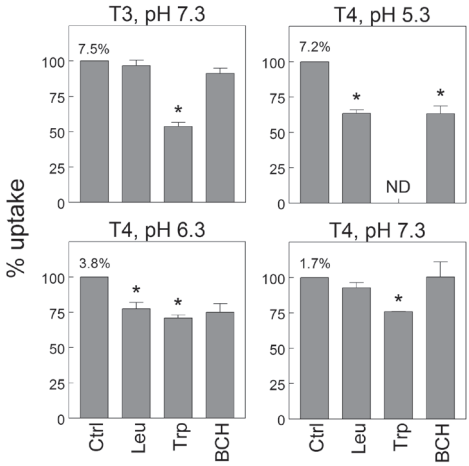


Figure 6: Effects of 1 mM amino acid transporter substrates on the uptake of 1 nM [^{125}I]T3 and [^{125}I]T4 during incubation in PBS with 0.1% glucose and 0.1% BSA, pH 6.3 or 7.3. Ctrl, control; Leu, leucine; Trp, tryptophan; BCH, 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid. Mean ± SEM, n=3. * $P<0.05$ vs. Ctrl. ND, not determined. Numbers above the Ctrl bar represent %T3 uptake in the absence of competitors.

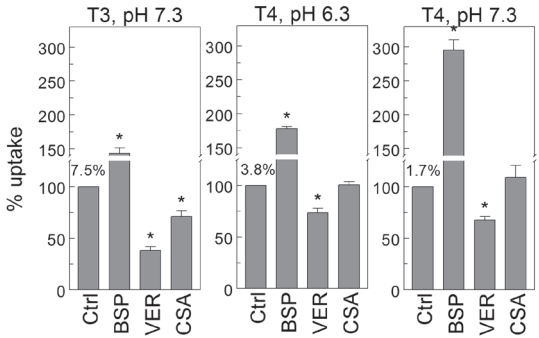


Figure 7: Effects of 1 mM OATP and MDR transporter substrates on the uptake of 1 nM [^{125}I]T3 and [^{125}I]T4 during incubation in PBS with 0.1% glucose and 0.1% BSA, pH 6.3 or 7.3. Ctrl, control; BSP, bromosulphthalein, VER, verapamil, CSA, cyclosporine A. Mean ± SEM, n=3. * P<0.05 vs. Ctrl. Numbers above the Ctrl bar represent %T3 uptake in the absence of competitors.

T3 uptake was not inhibited by 1 mM of the L-type amino acid transporter specific compounds BCH and Leu (Figure 6). However, T3 uptake was inhibited by substrates for T-type amino acid transporters (26), *i.e.* by 47% with 1 mM Trp (Figure 6), by 15% with 1 mM Phe and by 18% with 1 mM Tyr (not shown). Uptake of T3 was also inhibited by 62% with 0.1 mM VER and by 29% with 0.1 mM CSA (Figure 7).

BSP is a prototypic substrate for OATPs and MDR related efflux transporters (27). Remarkably, addition of 0.1 mM BSP resulted in a marked increase in T4 uptake by 195% at pH 7.3 and by 78% at pH 6.3, and an increase in T3 uptake by 43.6% (pH 7.3). A smaller BSP-induced increase in T4 uptake was also observed in the absence of BSA (38% at pH 7.3; not shown).

The Na-independent uptake of T4 by Caco2 cells and its inhibition by BCH and Leu suggested the involvement of L-type amino acid transporter(s) LAT1 or LAT2. LAT1 (SLC7A5) and LAT2 (SLC7A8) are the light chains which form functional heterodimeric transporters with the common heavy chain CD98 (SLC3A2) (28)*)* Therefore, we tested T4 uptake in COS1 cells co-transfected with LAT1 or LAT2 and CD98. T4 transport was not increased in cells transfected with LAT2, compared with cells transfected with empty vector (29). LAT1-transfected cells, however, showed pH-dependent uptake of T4, which was inhibited by BCH (Figure 8), in agreement with the Caco2 cell studies. In the absence of BSA, T4 uptake increased, but pH dependence remained intact. LAT1 also showed significant pH-dependent transport of T3 (Figure 8).

RT-qPCR analysis demonstrated marked expression of LAT1, LAT2 and MCT10 in Caco2 cells after 4 and 21 days of culture. Expression of LAT1 was 42% lower at 21 days than at 4 days of culture (Figure 9), LAT2 and MCT10 expression increased by respectively 21% and 16% at 21 days.

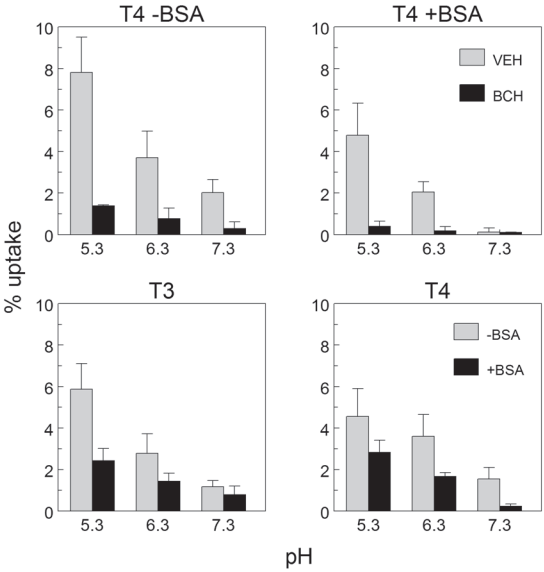


Figure 8: Effects of pH and BCH (1 mM) on uptake of [^{125}I]T4 or [^{125}I]T3 (1 nM) by COS1 cells transfected with LAT1 after incubation for 30 min in PBS with 0.1% glucose and without or with 0.1% BSA. Mean ± SEM (n=3).

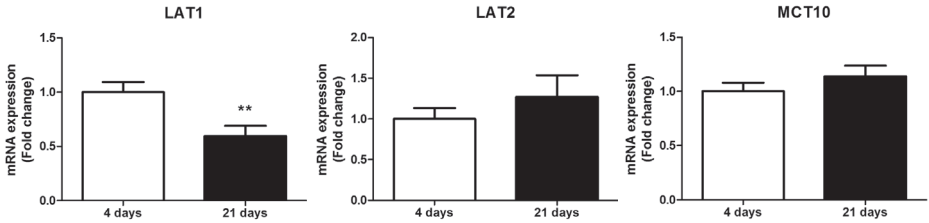


Figure 9: Expression of Thyroid hormone transporters LAT1, LAT2 and MCT10 in Caco-2 cells after 4 days and 21 days culture. MCT8 was not expressed. N=4. **p<0.01 t test

DISCUSSION

We used Caco2 cells as a model to explore TH transport characteristics in intestinal cells. Both T4 and T3 uptake by Caco2 cells increase with time, are saturable and Na^+-independent, implying the involvement of Na^+-independent transporters in these processes. Furthermore, T4 uptake is highly pH dependent, being markedly higher at pH 5.3 than at neutral pH. This is in contrast with the pH independence of T3 uptake and may be explained by the different effects of pH on the T3 and T4 molecules. The pK value of the phenolic hydroxyl group of T4 is ~6.5 and that of T3 ~8.5. This implies that this part of the T4 molecule is largely neutral at pH 5.3, whereas it largely exists as the phenolate anion at pH 7.3. In contrast, the phenolic hydroxyl group of T3 does not dissociate if pH is increased from 5.3 to 7.3, and thus remains largely neutral. However, it could also mean that different transporters are involved in T4 and T3 uptake.

Inhibition studies showed that T4 uptake at acidic pH was inhibited by Leu and BCH. The latter is a prototypic substrate for L-type amino acid transporters which transport large aliphatic and aromatic amino acids such as Leu, Trp, Tyr and Phe. There are at least 4 L-type amino acid transporters, i.e. LAT1 (SLC7A5), LAT2 (SLC7A8), LAT3 (SLC43A1) and LAT4 (SLC43A2) (30, 31). Recents studies from our lab indicate that LAT1 transports both T4 and T3, whereas LAT2 only transports T3 (19). LAT1-transfected COS cells showed the same characteristics of T4 uptake as Caco2 cells, *i.e.* increased uptake at lower pH and inhibition by BCH.

Independent of pH, T4 and T3 uptake by Caco2 cells is inhibited by VER and Trp. VER was tested as an inhibitor of P-glycoprotein to investigate the possible role of this efflux transporter on cellular accumulation of T4 and T3 in Caco2 cells. We therefore expected possible stimulation of net T4 and T3 uptake, but instead we observed significant inhibition of T4 and T3 uptake by VER. We have recently demonstrated dose-dependent inhibition of T4 and T3 uptake by MCT8 as well as MCT10 by VER (Sanne Noort, Theo J. Visser, unpublished work). Both MCT8 and MCT10 facilitate T3 and T4 transport in a Na^+-independent and pH-independent manner, with MCT10 clearly prefering T3 over T4 as the substrate. The marked inhibition of T4 and T3 uptake by VER and aromatic amino acids,

but not by Leu or BCH at neutral pH, suggests the involvement of MCT10 in this process.

BSP was tested as a prototypic substrate for OATPs, and a decrease (not an increase) in TH uptake in the presence of BSP was expected, if OATPs were to be involved in TH transport. The same increased uptake of TH in the presence of BSP was seen in HepG2, JEG3, COS1 and SHSY5Y cells (data not shown). Possible explanations of the increased TH uptake with BSP include 1) inhibition of cellular TH efflux by BSP, b) inhibition of TH binding to BSA by BSP, and c) the existence of an exchange mechanism where intracellular BSP drives the uptake of extracellular TH.

It is unclear if T4 and T3 transport in Caco2 cells is representative of TH transport in human enterocytes. LAT1 and LAT2 are known to be expressed in Caco2 cells (32), which was confirmed in our study. LAT1 mRNA has not been detected in the human intestine (33, 34), but a recently published map of the human tissue proteome reports a high expression of the LAT1 protein in the small intestine (35). In murine small intestinal cells LAT1 is located at the basolateral mebrane (36). To appreciate the function of LAT1 in the intestinal absorption of T4, it would be important to know if it is localized in the apical (luminal) or basolateral membrane. Although we demonstrated MCT10 mRNA expression in Caco2 cells, the MCT10 protein appears to be located in the basolateral membrane of human enterocytes (32, 37). This transporter, therefore, cannot be involved in the uptake of TH from the intestinal lumen but only from the blood. However, MCT10 appears to facilitate both cellular uptake and efflux of substrates. Therefore, it may also mediate the release of iodothyronines from the enterocytes into the blood.

In the present study we investigated TH transport in Caco2 cells as a model for TH transport in the human intestine. As absorption of T4 from the intestinal tract is a prequisite in the treatment of hypothyroidism, we chose this human cell line as a model instead of using cells from animal models. It is difficult to assess to which extent transport by Caco2 cells indeed represents transport in human enterocytes *in vivo*. Therefore, extrapolation of findings in Caco2 cells to human physiology may have limitations. However, Caco2 cells are commonly used to study transport

of various drugs in the small intestine (22). Therefore, it is generally accepted that these cells may serve as a model for transport processes in the human intestine.

In conclusion, we have provided evidence that there is transport of T3 and T4 in Caco2 cells and have described the transport characteristics. These characteristics are needed to understand the mechanisms of transport of TH by enterocytes. At neutral pH, MCT10 seems important in the transport of T3, and to a lesser extent T4. At acidic pH LAT1 seems to be important for T4 transport. Further studies are required to identify the transporters responsible for TH absorption in the human intestine.

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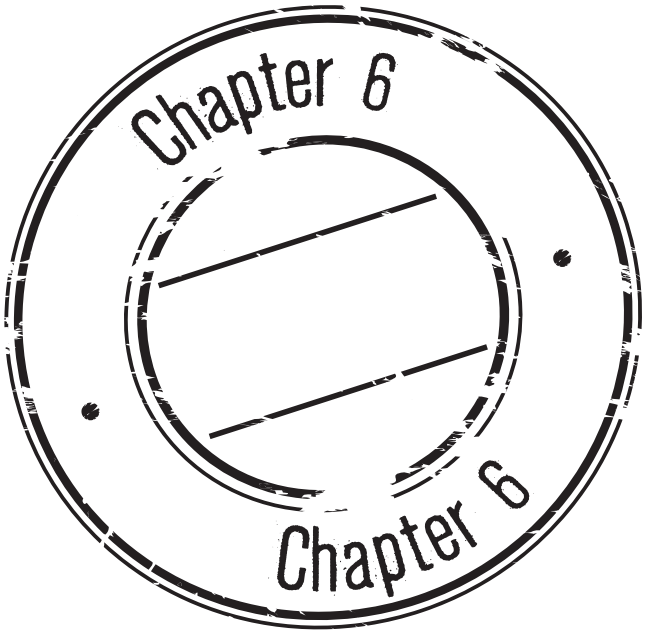
Supplemental Table 1. Primers sequence of the human THT

1	MCT8_Fw	CCTCTACTCCATGCTGCTA
2	MCT8_Rv	CGGTTGCTGTGATTCGG
3	MCT10_Fw	TGCTCTTCGTGTCCATGCTG
4	MCT10_Rv	GAGAGAGAACCTACCCATGC
5	LAT1_Fw	GAAGGGTGATGTGTCCAATCT
6	LAT1_Rv	GCAAAGAGGCCGCTGTATAA
7	LAT2_Fw	CATCGTAGGGAACATCATCGG
8	LAT2_Rv	GAGTTCAGCATAGCAGAGGG
9	PPIA_Fw	TTTCCAGGCCCCTTACCTCG
10	PPIA_Rv	CATGGTCAACCCTACCGTGT



Intestinal absorption of thyroxine

Review

N. Kelderman-Bolk, T.J. Visser, A. Berghout

Submitted

ABSTRACT

An adequate absorption of levothyroxine (LT4) by the intestine is an essential part of the treatment of hypothyroid patients. In the last decades research has provided more information on the location and mechanism of LT4 absorption in the gut. In this review we describe the available data on LT4 absorption and factors interfering with this absorption. To comprehend how a hypothyroid patient can best be treated with LT4, the mechanism of T4 uptake by the enterocyte and the entero-hepatic cycle of T4 and T3 are first described. In vitro experiments in Caco2 cells (a colon carcinoma cell line frequently used to study absorption in small intestinal cells) show the characteristics of the thyroid hormone transporters in the gut, and demonstrate the importance of an acidic environment for LT4 uptake. This explains why conditions impairing gastric acidity result in decreased absorption of T4. Interference of LT4 uptake by gastrointestinal disorders, several drugs and food is described next. Because of these interferences the timing of LT4 intake is important. Studies show that LT4 should be ingested on an empty stomach, where bedtime administration is an alternative to morning ingestion. Finally, pseudo-malabsorption (non-compliance) is often a diagnostic challenge. A guideline is presented how to carefully investigate this clinical problem.

INTRODUCTION

The absorption of T4 by the intestinal tract is an essential step in the treatment of hypothyroid patients, to adequately treat well-known hypothyroid symptoms (1, 2) and reach stable thyroid hormone levels. To reach normal T4 and T3 levels, hypothyroid patients are treated with synthetic T4 (levothyroxine, LT4), that is converted into T3 after absorption. Not only in hypothyroid patients but also in healthy euthyroid people T4 absorption is important, as it constitutes an important step in the entero-hepatic cycle of the hormone. During the last decades more information has become available on the location and the mechanism of T4 absorption by the intestine, and the factors influencing this absorption. We will present an overview of the available data of the research in this rapidly expanding field.

SEARCH STRATEGY

Pubmed database was searched for the terms ("Thyroxine"[mesh] OR Thyroxine[tiab] OR Thyroxin[tiab] OR "L-Thyroxine"[tiab] OR "L Thyroxine"[tiab] OR "Thyroid Hormone"[tiab] OR "Thyroid Hormones"[tiab] OR "thyroid treatment"[tiab] OR Levothyroxine[tiab] OR "L-T4"[tiab] OR LT4[tiab]). The results were cross-referenced with keywords relevant to the subsections. Additional references were identified from review articles and original research articles.

INTESTINAL ABSORPTION AND ENTERO-HEPATIC CYCLE

To understand the factors influencing the absorption of synthetic T4 (LT4) in hypothyroid patients, the mechanism of T4 absorption in the small intestine will first be described. Currently, it is known that the absorption of oral T4 across the mucosa takes place in the small bowel (3, 4). The location of T4 absorption was studied around the time that LT4 became commercially available (1970). In 1968 Hays wondered why athyreotic people needed 300 µg thyroxine to become euthyroid, while the normal thyroid gland secretes only about 80 µg of T4. As she suspected incomplete absorption of the oral thyroxine, she studied the

absorption of T4 by a double-isotope technique (3). T4 labelled with ^{125}I was administered orally simultaneously with intravenous T4 labelled with ^{131}I. The resulting serum $^{125}I/^{131}I$ ratio indicated a mean intestinal absorption of 71.1 +/- 9.0%. Later studies, including non-isotopic studies, confirmed an absorption of 60-80% after oral administration (4). T4 absorption occurs within the first 1-3 h of ingestion (5) and is localised mainly in the jejunum and the ileum (6). These findings seem to be in agreement with the experience of hypothyroid patients, who needed higher doses of LT4 after jejuno-ileal bypass surgery or other bowel resection. (7-9)

After absorption, T4 is transported to target tissues where it is further metabolised Peripheral thyroid hormone metabolism is mediated mainly by the three deiodinases (D1, D2 and D3), that catalyse the inner ring and/or outer ring deiodination of the different iodothyronines (10). D1 and D2 remove an iodine from the outer ring of the iodothyronine molecule, converting the biologically inactive prohormone T4 to the bioactive T3.

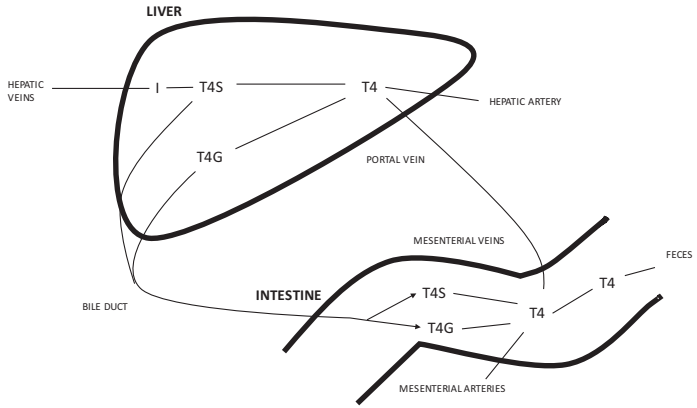


Figure 1 Metabolism of T4 by deiodination, sulfation and glucuronidation(adapted from thesis W.W. de Herder).

In addition, alternate pathways of thyroid hormone metabolism exist. These include conjugation of the phenolic hydroxyl group, decarboxylation and deamination of the alanine side chain and ether-link cleavage

(minor pathway) (11, 12). Conjugation of T3 and T4 is important in the enterohepatic cycle of thyroid hormones (Figure 1). Conjugation with sulfate or glucuronic acid increases the water-solubility of compounds, facilitating their biliary or urinary excretion. The sulfotransferases play a prominent role in the regulation of thyroid hormone metabolism, since sulfation of T3 and T4 accelerates their deiodination by D1 to the inactive metabolites rT3 and T2 (13). Glucuronidation of iodothyronines by UDP-glucuronyltransferases primarily occurs in the liver. The glucuronidated iodothyronines (T3G and T4G) are excreted in the bile and ultimately disposed of with the feces (14, 15). T3G and T4G, however, also serve as a thyroid hormone reservoir, as deconjugation back to T3 and T4 occurs in the intestinal lumen, catalysed by β-glucuronidases from intestinal bacteria (16, 17). Through intestinal absorption of the recovered T3 and T4, the hormones re-enter the portal circulation and are again available to the liver. Where and how the absorption of the recovered T3 and T4 takes place is unclear.

T4 TRANSPORT IN INTESTINAL CELLS

Around 1990, Hays and DiStefano concluded that T4 uptake is equally distributed over the entire intestinal tract and takes place by simple diffusion (6, 18). Although the side chain of iodothyronines is hydrophilic, it was assumed that the lipophilic aromatic part of T3 and T4 could diffuse through the lipid bilayer of the intestinal cell membranes. Studies of thyroid hormone uptake in other tissues however, show clearly that thyroid hormone traverses the cell membrane mainly with the help of transporters, indicating an active process (19).

Thyroid hormone uptake has different characteristics across cell types, with regard to ligand specificity, energy (ATP) dependence, Na^+-dependence and interaction with various compounds (20). Several thyroid hormone transporters have become known over the years, including Na^+/taurocholate cotransporting polypeptide (19), fatty acid translocase (21), multidrug resistance-associated proteins (22), L-type amino acid transporters (23), and members of the organic anion-transporting polypeptide (OATP) family (24) and monocarboxylate transporter (MCT) family (25). Some of the known thyroid hormone transporters have been localized in the small intestine,

including OATP1A2 (26), OATP2B1(27), MCT10, LAT1 and LAT2 (28). Recently we characterized the transport of T4 and T3 using the human colorectal adenocarcinoma cell line Caco2 as a model. (Kelderman-Bolk, submitted). This cell line is morphologically and functionally similar to human small intestinal epithelial cells making it a useful model for intestinal absorption of various compounds (29).

T4 uptake by Caco2 cells turned out to be Na^+ independent, but highly dependent on pH, T4 uptake being markedly higher at pH 5.3 than at pH 7.3. Kinetic studies with increasing concentrations of substrate showed saturation of T4 transport at pH 5.3 (with an apparent Km value of about 10 μM), which confirms that transporters are involved in the transport of T4 over the intestinal wall. The characteristics of T4 transport in Caco2 cells were further studied by testing the effects of prototypic ligands for the known thyroid hormone transporters. At acidic pH, T4 uptake is inhibited by prototypic ligands for the L-type amino acid transporters (leucine and 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH)), suggesting that T4 is transported in Caco2 cells by an L-type amino acid transporter (LAT 1 or 2) at low pH. Transporters of the OATP family are also known for pH-dependent transport (30), but our experiments suggest that OATPs have no role in intestinal uptake of T4, or that Caco2 cells are not a good model to identify human intestinal T4 transporter.

T3 uptake by Caco2 cells is both Na^+- and pH independent, and thus seems to be mediated by another transporter than T4 uptake. Inhibition studies indicate that T3 uptake is mediated largely by a T-type amino acid transporter, most likely MCT10. Further studies to clarify the exact mechanism of T4 and T3 uptake by intestinal cells should include knockdown experiments with siRNA, to test the importance of candidate transporters in T4 and T3 uptake.

FACTORS INTERFERING WITH THYROXINE ABSORPTION

There are several conditions in which the need for LT4 is increased. These include pregnancy (31) and the use of drugs that cause

hypothyroidism. This review, however, will only focus on conditions causing LT4 malabsorption in the gut.

Gastrointestinal disorders

The most apparent disorders where LT4 absorption is impaired, are in patients with a *resection* of part of the bowel or after an intestinal bypass. Because part of the gut is not available for T4 absorption, an increased dose of LT4 is required in these patients (7-9, 32). Besides that, conditions impairing gastric acidity and malabsorptive disorders can also affect the bioavailability of T4.

As the studies in Caco2 cells showed, T4 uptake is highly influenced by the acidity of the environment. This explains why conditions impairing gastric acidity result in decreased absorption of T4 (33, 34). In *Helicobacter pylori infection* of the stomach gastric acid secretion is impaired because of bacterial production of urease, which neutralises gastric pH. Centanni et al. (33) demonstrated an increased need of T4 in patients with H. pylori infection and *atrophic gastritis.* Medications that suppress gastric acidity may interfere with T4 absorption as well. However, conflicting results have been reported for the effects of *proton pump inhibitors* and the *H2-receptorantagonist* famotidine (33, 35-39). The apparent pH dependence of intestinal T4 absorption in vivo as well as of T4 uptake in Caco2 cells in vitro may be explained by the involvement of a pH dependent transporter, or by the effect of pH on the T4 molecule. The pK value of the phenolic hydroxyl group of T4 is ~6.5, which implies that this part of the T4 molecule is largely neutral at lower (acidic) pH values, whereas it largely exists as the phenolate anion at pH 7.3. In contrast, the phenolic hydroxyl group of T3 (pK ~8.5) does not dissociate if the pH rises to pH 7.3 and thus remains largely neutral.

In coeliac disease, lactose intolerance and parasitic infections, malabsorption of T4 leads to the need for an increased LT4 dose (40). Various cases of T4 malabsorption in *coeliac disease* have been reported (32, 40-42). It is hypothetised that partially undigested substances interact with LT4, making LT4 less available for absorption (40, 43). Patients with coeliac disease also have a different intestinal microbiota profile compared to controls (44), which could be another cause of LT4 malabsorption (45). Following treatment with a gluten-free diet the LT4 requirements often return back to normal. Coeliac disease and *atrophic*

gastritis occur frequently in patients with autoimmune hypothyroidism (46). Therefore, when a patient needs higher LT4 doses than expected, screening for coeliac disease and pernicious anemia (atrophic gastritis) is warranted. A small cohort study showed an increased need for oral LT4 in hypothyroid patients with *lactose intolerance* (47). In a case report T4 malabsorption in a patient with lactose intolerance improved with a lactose-free diet and lactose-free LT4 tablets (48). Seppel et al. described a case of a parasitic infection by *intestinal giardiasis,* causing T4 malabsorption (49).

Inflammatory bowel disease is mentioned in several review articles as a possible cause of T4 malabsorption (32, 43). However, no original studies describing this phenomenon are available to our knowledge.

Recently, two studies showed unchanged, or even improved absorption of LT4 after *sleeve gastrectomy, Roux-en-Y gastric bypass* and *bilio-pancreatic diversion* (50, 51). The authors conclude that the stomach, duodenum and upper jejunum are not important for LT4 absorption. This is in conflict with the earlier described results (7-9) . The authors speculate that bypassing the biliary secretions (containing glucuronized T4), changes in gut microbiota or reduced binding of LT4 to proteins normally present in the stomach, could explain the results. A better compliance, the weight loss or a diet change after surgery are other possible explanations for the presented results.

Food and drinks

Wenzel and Kirchsieper first reported on the impairment of LT4 absorption by simultaneous *food intake* (52), which was later confirmed by a study of Benvenga et al. (53). The latter study showed that maximum T4 absorption occurs between 30 and 60 minutes following LT4 ingestion, and that most T4 was absorbed within the first 90 minutes. When patients took LT4 15 minutes prior to breakfast, a delayed time to peak T4 absorption, reduced maximal absorption and decreased maximal increment in T4 absorption were seen. The results suggested an impairment in the early phase of the absorption. After separating breakfast and LT4 intake by at least 60 min thyroid hormone levels normalized, which led to the current recommendations to ingest LT4 60 min prior to breakfast.

A *fibre-enriched diet* also affects the bioavailability of LT4, as suggested by higher TSH levels and/or higher LT4 requirements (54). In vitro studies with various amounts of wheat bran showed a dose-dependent non-specific adsorption of LT4, explaining the reduced T4 bioavailability. No interference was found of the fibre supplements calcium polycarbophil or psyllium with LT4 absorption (55).

Several case-reports describe persistent hypothyroidism in treated patients consuming *soy* foods (56). The impact of soy on LT4 requirements appears to result from malabsorption, not from a systemic effect on the thyroid. This is concluded from experiments in the 1950's that demonstrated increased fecal excretion and decreased urinary clearance of LT4, in patients receiving soy compared to milk formula. The mechanism of decreased T4 absorption is unclear; it may be caused by sequestration of T4 in the intestine by soy, or interference with T4 uptake by a thyroid hormone transporter.

Espresso coffee was found to decrease the absorption of LT4 (57). In patients on LT4, elevated TSH levels decreased after separating coffee intake from LT4. In vitro binding studies indicated that coffee is able to sequestrate T4, explaining the reduced bioavailability. The study further showed that the intra-gastric pH is not changed by coffee.

To overcome the problems with LT4 absorption in patients drinking espresso coffee, a *soft gel capsule* containing T4 in glycerine has been developed that is unaffected by consumption of coffee (58). The authors suggest that this capsule might also overcome absorption problems in patients with gastrointestinal disorders. A liquid LT4 formulation has also been introduced in a few countries. An in vitro study measuring T4 by liquid chromatography-tandem mass spectrometry, showed that T4 is stable when this liquid LT4 formulation is added to various breakfast beverages (59). A clinical study showed no difference in serum thyroid parameters when liquid LT4 was consumed with breakfast or consumed 30 minutes before breakfast (60). A study in patients with impaired LT4 absorption by proton pump inhibitors, showed a correction of this malabsorption when patients switched to the oral solution LT4 in comparison with a LT4 tablet (61). The oral solution might resolve malabsorption of LT4 in many of the above described conditions.

Like coffee, fruit juices are also frequently consumed around breakfast time. Grapefruit juice increases the plasma concentrations of several drugs that are substrates for CYP3A4 through inhibition of this enzyme (62). However, *grapefruit juice* may decrease the plasma concentrations of other drugs, possibly by inhibiting uptake by intestinal transporters, in particular OATPs (63). The effect of grapefruit juice on LT4 absorption was studied in ten healthy subjects, showing limited effects on the pharmacokinetics of LT4: the amount of T4 absorbed was only slightly decreased, while t_{max} was not significantly prolonged (64). Cellular uptake of T4 is decreased by *orange juice* in vitro (unpublished work), apparently by binding of T4 to the fibres present in orange juice. To our knowledge, the effect of orange juice on the bioavailability of LT4 has not been studied in vivo.

Interestingly, a recent report showed that LT4 absorption was improved in patients with gastritis when the dose was ingested with water containing *vitamin C* (65). This again stresses the importance of an acidic environment for T4 absorption. The reduced acidity in gastrointestinal disorders could possibly be overcome by vitamin C supplementation.

Drugs

First, the formulation of LT4 itself and the *bioequivalence of different brands* need some attention, as there are concerns among clinicians that brand name LT4 and generic LT4 formulations are not clinically interchangeable. Since 1997 the quality of manufactured and distributed LT4 has improved by monitoring by the Food and Drug Administration (FDA). According to current guidelines, tablets should contain 95-105% of labelled content (66). Clinicians however have different experiences with interchangeability of LT4 formulations, and point out the inability of FDA pharmacokinetic testing, using supra-therapeutic doses, to predict clinical outcomes with significantly lower doses (67). A joint statement of The Endocrine Society and American Thyroid Association therefore still recommends that patients should not switch from LT4 brand or generic formulation, or should have their TSH retested after switching their LT4 preparation (68).

The list of several, often commonly prescribed, drugs that interfere with the bioavailability of LT4 has increased over the years (Table 1) (32).

Table 1 Drugs reducing the absorption of levothyroxine

Drug	Effect	Mechanism
Cholestyramine	Decreased absorption T4	Irreversible cholestyramine-thyroxine complex
Colesevelam	Decreased absorption T4	No in vitro study available
Ferrous sulfate	Decreased absorption T4	Insoluble ferric-thyroxine complex
Sucralfate	Conflicting data about possible decreased absorption T4?	Sucralfate-thyroxine complex or impaired gastric acidity
Aluminium hydroxide	Decreased absorption T4	Adsorption or complex forming or impaired gastric acidity
Calcium carbonate, citrate, acetate	Decreased absorption T4	Adsorption or impaired gastric acidity
Proton pump inhibitor	Conflicting data	Impaired gastric acidity
Raloxifene	Decreased absorption T4	No in vitro study available
Ciproxin	Decreased absorption T4	Interfering with thyroid hormone transport
Sevelamer hydrochloride	Decreased absorption T4	No in vitro study available
Chromium pilonate	Decreased absorption T4	No in vitro study available

For most drugs it is assumed that the reduced bioavailability of LT4 is caused by sequestration of T4 in the intestinal lumen or by decreased gastric acidity. For some, but not all, drugs the mechanism for the reduced bioavailability of LT4 has been studied in vivo and in vitro.

In1969 Northcutt reported on a decreased intestinal absorption of LT4 with the concomitant use of the bile sequestrant *cholestyramine* (69). In vitro studies showed that 50 mg of cholestyramine bound at least 3000 µg LT4. The results from this study led to the recommendation to take LT4 and cholestyramine 4-6 hours apart. Interestingly, cholestyramine has also been studied for the treatment of thyrotoxicosis in Graves' disease (70, 71). The excess of thyroid hormone in the enterohepatic circulation in patients with Graves' disease is successfully reduced by cholestyramine. When cholestyramine is added to the treatment with propylthiouracil (PTU), this results in a faster normalisation of thyroid hormones than treatment with PTU alone (70, 71). *Colesevelam hydrochloride,* a sequestrant with apparently higher affinity for bile

acids, also markedly reduces LT4 absorption. The optimal time interval between ingestion of LT4 and colesevelam has not been studied. *Colestipol,* another bile sequestrant, reduces LT4 absorption in rats (72) but not in humans (73).

Ferrous sulphate decreases LT4 absorption, resulting in an increase in serum TSH (74). In vitro spectrophotometric studies showed that insoluble Fe^{3+}-T4 complexes are formed, probably resulting in a reduction in intestinal LT4 absorption. Extrapolating the results from other studies on drug interactions with iron supplements (75), the authors suggest to separate the intake of LT4 and ferrous sulphate by at least 2 hours.

Conflicting results have been reported for the effect of *sucralfate* (used in reflux disease and gastritis) on LT4 absorption. Reduced and delayed absorption of LT4 was found in one study, which disappeared when the medications were taken 8 hours apart (76). These findings could be explained by binding of T4 by sucralfate as found in earlier in vitro experiments (77). Other studies, however, do not confirm the reduced bioavailability of LT4 by sucralfate (78, 79).

LT4 absorption is decreased by *aluminium hydroxide* (80-82). In vitro experiments show incremental LT4 adsorption with increasing concentrations of aluminium hydroxide, suggesting intraluminal sequestration of T4 by aluminium hydroxide. LT4 absorption may also be affected by formation of Al^{3+}-T4 complexes.

The bioavailability of LT4 is also reduced by *calcium carbonate* (83, 84). In vitro studies showed significant adsorption of T4 to calcium carbonate. A later study also showed impaired LT4 absorption with *calcium citrate* and *calcium acetate* (85).

The above-mentioned medications like calcium carbonate, aluminium hydroxide and sucralfate also interfere with gastric pH and/or acid secretion. As the in vitro studies in Caco2 cells have shown, pH is important for T4 uptake by intestinal cells. Low gastric acidity may thus contribute to the reduced bioavailability of LT4 with concomitant use of these drugs.

As described above, there are conflicting reports on the effects of *proton pump inhibitors* (PPI) on LT4 absorption (33, 35-39). As with the above-mentioned medications, low gastric acidity induced by proton pump inhibitors may reduce LT4 absorption. Two retrospective studies and 2 prospective observational studies in a small group of patients showed that the LT4 daily dose had to be increased by 20-37% to reach the same therapeutic effect after starting PPI treatment (33, 36, 39). In two other studies LT4 absorption was unaltered with the use op PPIs during 1 week (37, 38). This may suggest that only chronic oral PPI therapy is associated with decreased LT4 absorption.

Raloxifene, a selective estrogen receptor modulator, causes T4 malabsorption when administered together with LT4, but not when the drugs are taken several hours apart (86). The mechanism for this reduced T4 absorption is unknown. Raloxifene is not known to adsorb or form complexes with other medications. The possibility of interference with a thyroid hormone transporter should be considered.

A more recent paper showed a blunted rise in serum T4 after ingestion of 1 mg LT4 together with the phosphate-binding drug *sevelamer* or the nutritional supplement *chromium picolinate* (87). The authors suggested that adsorption of LT4 to sevelamer or chromium, or alterations in mucosal transport processes may be the cause of the decreased T4 absorption. In vitro experiments have not been performed to investigate these possible mechanisms.

Recently, reduced absorption of LT4 by the antibiotic *ciprofloxacin* has been described (88) which cannot be explained by binding of T4 to ciprofloxacin or reduced gastric acidity. The interaction by ciprofloxacin with a thyroid hormone transporter in the intestinal wall could offer an alternative explanation. Goldberg et al. show that the reduced absorption of LT4 by ciprofloxacin is consistent with inhibition of intestinal T4 transporters, possibly members of the OATP family, in particular OATP1A2 (89). Our in vitro experiments with Caco2 cells, however, showed no decreased T4 uptake in the presence of the prototypic OATP ligand BSP. OATP1A2 was shown to be present in Caco2 cells in a study on transport of another quinolone antibacterial agent levofloxacin (90).

Most of the studies on T4 malabsorption by other medications were performed before the importance of T4 transporters was established. Additional studies clarifying the role of specific intestinal (and liver) transporters in LT4 absorption, will also result in a better understanding of the interaction of LT4 with other drugs.

TIMING OF THYROXINE INGESTION

Because of all the above mentioned factors possibly interfering with LT4 absorption, the moment of LT4 ingestion is important to maintain optimal and stable absorption. Patients are generally advised to take LT4 on an empty stomach. In 1977 Wenzel et al first reported on the influence of food on the absorption of LT4, and showed that absorption was significantly better in a fasting state than with simultaneous food intake. (52) The study of delayed T4 absorption with simultaneous food intake by Benvenga et al. (53) led to the current recommendations to ingest oral LT4 60 min prior to breakfast. In daily practice this stringent recommendation could potentially affect compliance. Furthermore, there are circadian differences in gastrointestinal function that could improve LT4 absorption when taken at night. Basal gastric acid secretion is highest in the late evening and lowest in the morning (91) and, as mentioned above, a low intestinal pH improves T4 absorption. In addition, bowel motility decreases at night (92), resulting in a more prolonged exposure of the intestinal wall to T4, which could further improve LT4 absorption. In the last couple of years a number of randomized trials have studied various timing options for LT4 intake and their effect on thyroid hormone parameters, variability of TSH values and quality of life (QOL). Bach-Huynh et al. showed that T4 is best absorbed when taken on an empty stomach, with at least 60 minutes separating LT4 and food intake (93). In a three-period crossover design they compared LT4 intake 1) 1 hour before breakfast, 2) with breakfast or 3) at bedtime at least 2 hours after the last meal of the day. TSH concentrations were higher when LT4 was taken at bedtime (TSH 2.19 ± 2.66 mU/l) and with breakfast (TSH 2.93 ± 3.29 mIU/l), compared with ingestion 60 min before breakfast (1.06 ± 1.23 mU/l). The non-fasting regimens were also associated with more variable serum TSH concentrations. No changes in FT4 and T3 concentrations were seen. As the authors point out, only 19% of the eligible patients agreed to

participate, which may have resulted in a selection of patients who were rigorous in adhering to their medication, making the results less generalisable. In every day life, most patients find it difficult to postpone breakfast for 60 minutes after LT4 ingestion.

In a pilot study, later confirmed by a randomized double-blind crossover trial, we showed that LT4 taken at bedtime resulted in better thyroid hormone parameters, than when taken 30 min before breakfast (94, 95). The pilot study also showed that the circadian pattern of TSH secretion remained intact with bedtime ingestion. This was an important finding, regarding the time of blood sampling for the analysis of the TSH levels. In the randomized trial, patients (n=90) were instructed to take 1 capsule in the morning and 1 capsule at bedtime (one containing LT4 and one containing placebo), with a switch after 3 months. Results showed that when LT4 was taken at bedtime, serum TSH levels decreased 1.25 mIU/l and FT4 and T3 levels increased compared to the morning intake. The higher TSH level with the morning LT4 intake suggests that an interval of 30 min between LT4 ingestion and taking breakfast may be too short to prevent interference with gastrointestinal absorption of LT4.

Finally, a recent randomized, open-label crossover study compared LT4 intake at least 60 minutes before breakfast with LT4 intake during breakfast (96). TSH level was higher when LT4 was taken with breakfast than on an empty stomach (TSH 2.89 vs 1.90 mIU/l). Intake with breakfast also resulted in more variability in TSH levels, which confirm the results of Bach-Huynh et al. (93).

In conclusion, all studies of the timing of LT4 intake conclude that absorption is maximal and most stable when the drug is taken on an empty stomach, with at least 60 minutes between tablet ingestion and food intake. LT4 intake at bedtime is a good alternative to morning intake, provided that LT4 is taken on an empty stomach. This regimen might be more convenient for patients that cannot postpone breakfast for 60 minutes in the morning.

PSEUDO-MALABSORPTION

A relatively common problem in endocrine practice is persistent elevation of TSH in patients treated for hypothyroidism (97). The average replacement dose of LT4 varies from patient to patient, but is usually between 1.6-1.8 µg/kg. If much higher doses are required without adequate normalization of the thyroid hormone parameters, one should question the cause of this treatment failure. Rare causes like thyroid hormone resistance, or a TSH secreting pituitary adenoma show high TSH in combination with elevated FT4 levels. However, when despite high doses of LT4 raised TSH levels are seen with low FT4 levels, malabsorption or pseudo-malabsorption (non-compliance) are the most common causes. First, compliance should be checked with the patient. LT4 should be taken on an empty stomach and intake of food should ideally be avoided for 60 minutes. Furthermore, drugs known to interfere with LT4 absorption/pharmacokinetics (Table 1 + 2) should be ingested with the appropriate time between LT4 and the drug(s). When these instructions do not improve thyroid hormone levels, all causes of LT4 malabsorption should be excluded (Table 2).

Table 2 Factors interfering with LT4 treatment

Gastrointestinal disorders	Coeliac disease, H pylori gastritis atrophic gastritis, lactose intolerance, infections (giardia lambia)
Previous gastrointestinal surgery	Jejuno(ileal) bypass, short bowel
Liver disease	Cirrhosis, obstructive liver disease
Pancreatic disease	Pancreatic insufficiency
Interfering medication- malabsorption	Table 1
Interfering medication- change pharmacokinetics	Carbamazepine, phenytoin, phenobarbital, rifampicin, amiodarone, estrogen therapy
Dietary interference	Interval between LT4 and food intake (minimum 60 min), fibre-enriched diet, soy products, coffee
Pregnancy	Increases need LT4

If all malabsorptive disorders are excluded, pseudo-malabsorption is the most probable cause. To prove this, a LT4 absorption test can be performed in a clinical setting. A single high dose of LT4 1000-2000

µg should be ingested by the patient under supervision of a nurse, to check proper ingestion and prevent surreptitious regurgitation (98, 99). TSH and FT4 levels should then be monitored over time (0, 2, 4 and 6 hours after ingestion). No well-established standard is available to which individual patient results can be compared. However, from previous case reports and data on pharmacokinetics of LT4, it is known that peak absorption takes place 2-4 hours (5) after ingestion and FT4 levels should rise 50-100% above basal level (98, 99). Normalization of FT4 levels also proves the absence of a malabsorptive disorder (100).

CONCLUSION

A stable and optimal absorption of LT4 is important in the treatment of patients with hypothyroidism. As described, absorption can be decreased by gastrointestinal disorders, including malabsorptive disorders and disorders impairing gastric acidity. From in vitro experiments in Caco2 cells, the importance of a low pH for T4 uptake by intestinal transporters became apparent. Several, often commonly prescribed, drugs decrease the absorption of T4. This happens either by adsorption of LT4 to the interfering drug, by impaired gastric acidity, or by competition with a still unknown thyroid hormone transporter in the intestinal cell.

To ensure optimal absorption of LT4, patients are advised to take LT4 on an empty stomach, with at least 60 minutes between LT4 and food ingestion. Bedtime LT4 intake is a more convenient alternative to morning intake, provided that LT4 is taken on an empty stomach. When patients have persistent elevation of TSH levels, despite high doses of LT4, malabsorption by gastrointestinal disorders or drugs should be excluded. When excluded, pseudo-malabsorption (non-compliance) is the most common cause, and a supervised high-dose LT4 absorption test can be performed.

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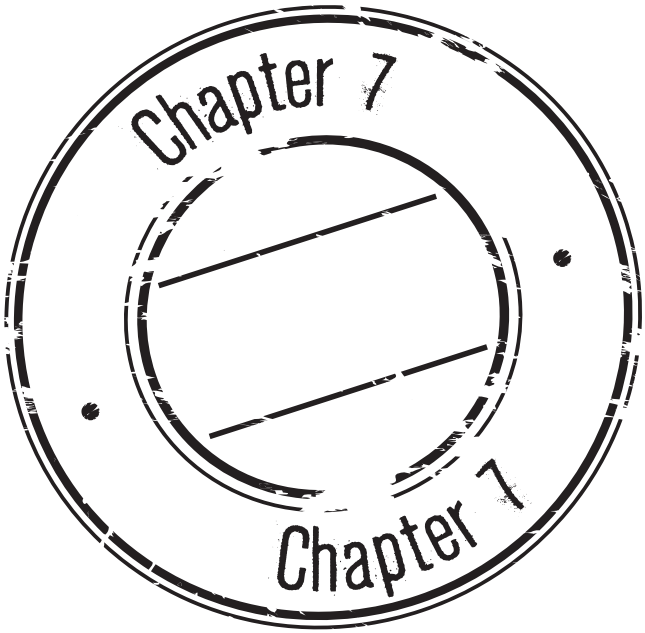

General Discussion

INTRODUCTION

The prevalence of primary hypothyroidism keeps increasing, with iodine deficiency being the most common cause worldwide, and autoimmune thyroiditis being the most prevalent cause of hypothyroidism in developed countries with sufficient iodine intake. Treatment with synthetic T4 is available and seems to be the best substitute for the normal thyroid function. It has become increasingly clear, however, that treated hypothyroid patients regularly have remaining complaints. Nowadays, patients are becoming more assertive, assemble in patient platforms and demand an explanation for their reduced quality of life (QOL). Often they gather information on hypothyroidism on the internet and suggest possible alternative treatments. A better understanding of thyroid hormone (TH) absorption in the intestine and the reasons for the reduced QOL would help in the treatment of hypothyroid patients.

The problem in hypothyroidism is the decreased levels of serum T4 and T3, which cause a generalized slowing of metabolic processes in the human body. This results in complaints of cold intolerance, constipation, weight gain and hair loss. Also atherosclerosis, hypertension, dyslipidemia and depression can occur (1, 2). It is therefore important in the treatment of hypothyroidism to substitute the lack of TH in a way that mimicks the normal thyroid function as much as possible. Several studies on the best substitution therapy for hypothyroidism have shown that TH levels and complaints improve best by the treatment with synthetic T4, levothyroxine (LT4) (3). Therefore, international guidelines (4, 5) prescribe LT4, as the treatment of choice for patients with hypothyroidism. As several factors (food, drugs etc) can decrease LT4 uptake (6), instructions are that the patient should take LT4 in the morning on an empty stomach 30-60 minutes before breakfast. These strict instructions are not only inconvenient, frequently patients also have remaining complaints, even if TH levels have normalized.

In this thesis we 1) present bedtime LT4 intake as a good and more convenient alternative to morning intake, 2) describe the circadian rhythm of TSH, FT4 and T3 with morning and bedtime LT4 intake, 3) investigate the cause of reduced quality of life (QOL) in treated hypothyroid patients, 4) characterize TH transport in a intestinal cell model and 5) describe the factors influencing LT4 uptake by the intestine.

LT4 INTAKE IN THE MORNING

Shortly after the introduction of commercially available synthetic LT4 in 1970, several studies appeared on the interference of intestinal diseases, and simultaneous intake of food and specific drugs with LT4 uptake (6). The package leaflet therefore states that LT4 should be taken on an empty stomach in the morning. Dutch prescibing information states that LT4 should be taken 30 minutes before breakfast, and American prescribing information advises an interval of 60 minutes before taking any food. Furthermore, other interfering drugs should be taken 2-6 hours after LT4 ingestion. For patients these instructions can be inconvenient because of their (work) schedule, concurrent illnesses (*e.g.* diabetes) and other drugs that should be taken before or with meals. This made us wonder if LT4 could be taken at another time of day, when the stomach is empty, for example at bedtime. When several patients in our outpatient clinic where positive about switching to bedtime LT4 intake, with improvement of their thyroid hormones, we decided to study the timing of LT4 intake in a randomized trial. We were interested in the effect on TH levels, but also in the possible changes in vital parameters (heart rate, body weight, blood pressure) and the effect on QOL.

LT4 INTAKE AT BEDTIME

The first study we performed on bedtime LT4 intake in 11 patients (**Chapter 2**) already showed a significant improvement in TH levels compared to morning LT4 intake (7). As this was not a blinded study, a better compliance with bedtime intake could have influenced the results. Therefore, it was necessary to perform a larger, randomized double-blind trial, to confirm the results of the pilot study. The cross-over design with capsules prepared by our hospital pharmacy (placebo and LT4), made it possible to study the effect of switching from morning to bedtime LT4 intake (or vice versa) with patients (n=90) being their own controls.

The results described in **Chapter 3** showed that TSH levels decreased, and T4 and T3 levels increased with bedtime LT4 intake compared to morning LT4 intake (8). A possible explanation could be that basal gastric acid secretion is highest in the late evening and lowest in

the morning (9). In addition, bowel motility decreases at night (10), resulting in a more prolonged exposure of the intestinal wall to T4, which could further improve LT4 absorption. Also, the observation that serum FT4 increased after LT4 intake in the morning, but not with the bedtime intake in the pilot study, could point to the fact that there is a slower release of T4 with bedtime intake.

Five other studies examined the effect of different timing of LT4, especially in relation to food intake (11-15). A case study in 1995 in four patients showed that consuming breakfast within 20 minutes after LT4 intake resulted in higher TSH levels than when the same patients had breakfast 60 minutes after taking their LT4 (11). This small study led to the current recommendations in prescribing information to take LT4 60 minutes before breakfast. A retrospective study in elderly patients comparing LT4 intake at 9 a.m. with midnight intake (both postprandial) showed no difference in TSH levels (13). A parallel design study in newly diagnosed hypothyroid patients (with a mean TSH of 80 mIU/l at the start of the study) showed no significant difference in TSH level with evening dosing compared to morning dosing after 12 weeks of LT4 treatment. (14). Probably a steady state had not yet been reached in this trial. Only two other studies had a cross-over design like our study. An American trial, published around the same time as our study, compared 3 different times of LT4 intake (at least 1 hour before breakfast, with breakfast or at bedtime) for 8 weeks (15). The mean TSH levels seen with these different regimens were 1.06, 2.93 and 2.19 mIU/l respectively. LT4 intake on an empty stomach in the morning, therefore, showed a lower TSH level than bedtime intake in this study, probably due to the longer time between breakfast and LT4 intake (60 min versus 30 minutes in our study). A third cross-over study compared LT4 intake in the morning before or with breakfast, and showed more stable TSH values with intake on an empty stomach (12). Thus only 2 studies comparing morning and evening LT4 intake (including ours) had a randomized controlled cross-over design. The main conclusion is that LT4 has to be taken on an empty stomach, with as much time as possible between LT4 intake and the ingestion of food or other drugs. If a patient has an empty stomach at bedtime, LT4 intake at that moment could be a good and perhaps more convenient alternative to morning intake.

The performed studies influenced the guidelines on treatment of hypothyroidism. The Dutch guideline on thyroid disorders (Schildklierfunctiestoornissen 2012) states that bedtime LT4 intake is an alternative for morning intake. Two guidelines on hypothyroidism published by the American Thyroid Association (ATA) (4, 16), both mention our randomized trial and comment on the best time to take LT4. In the most recent guideline of December 2014 focusing on the treatment of hypothyroidism (4), the ATA incorporated a separate paragraph 'Timing of levothyroxine administration' describing the six studies discussed above. The authors summarize that the conditions associated with better absorption (ordered from best to most impaired) are 60 min before breakfast, bedtime, 30 minutes before breakfast, and with breakfast. Bedtime intake is therefore considered superior to intake 30 minutes before breakfast, as Dutch pharmacies currently advise. The ATA also stresses that it is important to consider not only when LT4 absorption is optimal, but also to be aware of what timing promotes adherence. If it is more convenient for a patient to take LT4. at bedtime, this will improve compliance and wil result in better and more stable TH levels.

CIRCADIAN RHYTHM OF TSH

In healthy subjects, thyrotropin (TSH) released by the pituitary gland, exhibits a circadian rhythm. Serum TSH levels increase in the evening, reach a maximum in the early night and decrease during the night (17, 18). In addition to this nycthemeral pattern, repeated variably sized bursts of TSH release occur during the 24-hour period. TSH secretion is, therefore, partially pulsatile and partially basal. During the nocturnal TSH rise the burst frequency and amplitude increase. How TSH pulses are generated is not known, in contrast to the secretory pattern of other pituitary hormones (ACTH, GH, LH). What is known is that TSH secretion is under stimulatory control of thyrotropin releasing hormone (TRH) and the hormone leptin, gets negative feedback by T4 and T3 and is inhibited by the neurotransmitters somatostatin and dopamine (19). The circadian TSH rhythm is still present in mild hypothyroidism, disappears in overt hypothyroidism, but is restored with LT4 substitution (20). **Chapter 2** describes the 24 hour patterns of serum TSH, (F)T4 and T3 with morning and bedtime LT4 intake in 11 hypothyroid patients (7).

We confirmed the presence of a circadian TSH rhythm and showed that the pattern does not change when switching the time of LT4 ingestion to bedtime. Blood sampling in patients taking LT4 at bedtime can, therefore, still take place in the morning, as is usually done, without causing a systematic error in interpreting the results.

As was shown in previous studies (21), we demonstrated a transient peak in serum T4 and FT4 levels about 4 hours after morning LT4 administration. This peak was not seen, however, with bedtime LT4 intake, possibly explained by the reduced bowel motility at night (10), causing a slower release of T4 into the blood.

Our study showed a circadian pattern for FT4 and T4 both with morning and bedtime LT4. This pattern did change with bedtime LT4, showing a slightly decreased relative amplitude and a phase-advanced timing of their acrophase (earlier peak). Previous studies have shown conflicting data on diurnal rhythms in T4 and T3 (22, 23). Some publications suggest a relation between TSH and thyroid hormone secretion (23-25), but others do not (26), which may be explained by different experimental conditions and analytic tools. Changes in TH levels (total T4 and T3) could be explained by changes in serum protein levels effected by postural changes, but this does not explain the circadian pattern in FT4. The mechanism by which the circadian pattern of FT4 and T4 is influenced by bedtime LT4 administration is therefore not easily explained.

Furthermore, the bioactivity of TSH also shows a circadian rhythm, with less bioactive and differently glycosylated TSH at night (27). So, while TSH levels are higher at night, the bioactivity of the hormone is less. We did not study the influence of different LT4 administration times on the bioactivity of TSH.

In recent years the sensitivity and precision of TSH assays have improved. Also the mathematical tools to interpret the 24-hour TSH pattern are much better, with the use of deconvolution methods (to quantify significant pulses, half-lives and basal secretion) and approximate entropy (quantifying the pattern orderliness) (19). This has provided information about the influence of age, gender, BMI and sleep on the circadian TSH pattern. With these modern methods more

information could be gathered in future studies about the change in TSH pulse frequency, pulse shape and half-lives when changing the administration time of LT4. Studying the circadian patterns of TSH and thyroid hormones with different treatments of hypothyroidism (LT4, T3/T4 combination) could help to find the best way to mimick normal thyroid function.

QUALITY OF LIFE IN HYPOTHYROIDISM

Despite improved thyroid parameters with bedtime LT4, QOL did not improve in these patients. In general, in newly diagnosed hypothyroidism, symptoms and signs improve quickly after starting LT4. However, when the treatment target is reached, with TH levels within the reference range, patients frequently have remaining complaints. Clinicians encounter this problem in hypothyroid patients on a daily basis.

To find an explanation for the reduced QOL, we performed a post-hoc analysis on the collected data from our randomized trial on bedtime LT4 in hypothyroid patients, of which the results are described in **Chapter 4** (28).

A reduced QOL in these patients was confirmed when comparing the QOL scores with a healthy population. No relation was found between QOL and thyroid parameters, as other studies had already shown (29, 30). The parameter that did have a relation with QOL was BMI, with a higher BMI leading to a worse QOL. BMI in our population was indeed markedly higher than the BMI in the general Dutch population. The weight gain observed in patients with hypothyroidism is a result of the decreased metabolism. When treatment is started, however, most patients struggle to loose the gained weight, and often do not succeed. It is known that QOL is reduced in overweight people in general (31), and in hypothyroid people it seems to explain a large part of the reduced QOL. Therefore, the prevention of weight gain requires attention in the treatment of hypothyroid patients and in future studies.

Possible improvements could be aimed at diagnosing hypothyroidism at an earlier stage (for example with a screening program in a high risk

population), or adaptations in the treatment of hypothyroidism. A more rapid normalization of TH levels with a full starting dose of LT4 (1.6 µg/kg) would seem beneficial, but a randomized trial showed no change in QOL scores or body weight compared to a low starting dose (32). Aiming at a lower target TSH was also studied, but did not give an improvement of QOL scores (33). Also, TSH levels below the lower reference range are not recommended, because this increases the risk of atrial fibrillation (34) and osteoporosis (35). Studies on treatment with desiccated TH (36) or T4/T3 combination therapy (3, 37) showed no improvement in QOL compared to LT4 monotherapy. However, two studies did show a patient preference for T4/T3 combination therapy (37) or dessicated TH (36), a result possibly explained by its association with weight loss. Other studies have shown that LT3 monotherapy is associated with decreased body weight and improved lipid levels (38). This treatment, however, results in an unwanted reduction in TSH and FT4 levels.

Of course, not all patients with a reduced QOL are overweight, and BMI does not explain complaints in all hypothyroid patients. Recent studies (39) have linked reduced QOL in some hypothyroid patients to a type 2 deiodase polymorphism, DIO2-Thr92Ala, causing a reduced peripheral conversion of T4 into the active T3. In these specific patients, combination T4/T3 therapy is thought to be beneficial. *In vitro* studies, however, have failed until now to show a consistent functional effect of this DIO2 polymorphism(40). Further research needs to give more insight in this hypothesis.

As was discussed earlier, also T4 and T3 vary during the day, with the stimuli and inhibitory factors largely unknown. The current treatment with exogenous T4 does not reproduce endogenous serum T4 excursions or serum T3 rhythms. This could also explain the fact that T4/T3 combination therapy has not resulted in improved well being, as T3 taken once or twice daily may not replicate the physiologic nature of T3 secretion. Perhaps the development of a sustained release preparation could mimick the normal diurnal rhythm of T4 and T3 in the future.

An additional problem is that serum TH levels might not represent TH levels in different tissues, because of varying expression and function of TH transporters and deiodinases. A good marker of tissue TH status would, therefore, be helpful. Tissue biomarkers of thyroid

hormone include sex hormone binding globulin (SHBG), osteocalcin, urinary n-telopeptides, total cholesterol, low-density lipoprotein (LDL) cholesterol, lipoprotein(a), creatine kinase, ferritin, myoglobin, and enzymes such as tissue plasminogen activator, angiotensin converting enzyme (ACE) and glucose-6-phospate dehydrogenase (4). The results of RCTs have shown that SHBG (30) and total cholesterol (30, 41) are particularly affected by the administration of LT4. These biomarkers, however, are not sensitive and specific, or standardized, as was also seen with SHBG in our randomized study on bedtime LT4. One study on LT4 substitution showed that resting energy expenditure was altered by change in LT4 dose and correlated well with TSH (42). The additional value to TSH levels in the clinical setting however is not clear. Measurement of TH responsive gene expression is a means of assessing the impact of TH on various tissues. TH effects on rodent and cell models have been extensively studied (43). Measurement of gene expression, however, cannot be routinely used to asses TH status, as it would require invasive procedures such as taking tissue biopsies.

In conclusion, at this moment LT4 remains the best treatment for patients with hypothyroidism, but hopefully treatment in the future can be more customized to the individual hypothyroid patient, taking the individual setpoint and TH metabolism into accoount. To accomplish this, large prospective parallel studies are probably needed to study different treatment modalities rather than short-term cross-over studies.

INTESTINAL CELL MODEL STUDYING UPTAKE T4 AND T3

As mentioned before, the active hormone T3 needs to be transported into the cell to stimulate the nuclear TRs. As endogenous T4 and T3 lack in hypothyroidism, these hormones need to be substituted by oral administration. In the intestine, T4 has to be absorbed at the apical side of the enterocytes, transported through the cell, and released into the circulation at the basolateral side. TH transport in different tissues involves different transporters. In **Chapter 5** TH transport in intestinal cells is characterized using the colon carcinoma (Caco2) cell line as a model. This cell line in vitro undergoes spontaneous enterocytic

differentiation, and is therefore a frequently used model to study transport in the small intestine.

The results show that the uptake of T3 and T4 in enterocytes is regulated by different transporters. T4 uptake in the Caco2 cell is increased at a lower pH and inhibition studies with prototypic ligands for known TH transporters suggested that the L-type amino acid transporter LAT1 is involved in this T4 transport.

MCT10 seems to be important for T3 transport in Caco2 cells, because of the Na^+ and pH independence, inhibition of T3 uptake by MCT10 ligands, and reduced T3 uptake after silencing MCT10 with siRNA.

These data are important in the understanding of T4 and T3 uptake by intestinal cells. The study marks only the beginning, however, of our knowledge about TH transport by the intestine. We do not know if TH transport in Caco2 cells represents TH uptake *in vivo* in the human intestine. Although LAT1 and MCT10 are present in the human enterocyte, the expression and localization in the cell will influence the uptake *in vivo*. LAT1 is present in the small intestine (44), but its localization at the apical or basolateral membrane of the eneterocyte is not clear. MCT10 is at the basolateral membrane of the enterocyte (45), and may be important for the transport of T3 through the cell, but will need a different transporter at the apical membrane for uptake from the lumen.

Future research is needed to fully understand intestinal TH transport. Currently, our lab studies TH transport in Caco2 cells in transwells to get more information on transcellular transport in polarized cells. Experiments can also be performed using intestinal cells isolated from mouse models, as has been done for studies of the intestinal transport of other substances (46). Unfortunately, it is not yet possible to culture a stable cell line of human enterocytes, as biopsies of the small intestine contain different cell types. To make sure that future studies on thyroid hormone transport are conducted well and results from different groups can be compared the ATA published a guideline on how to investigate thyroid hormone transport in a cell model (43).

FACTORS INFLUENCING INTESTINAL T4 UPTAKE

Treating patients with hypothyroidism is a challenge with all the factors influencing the absorption of T4. The studies described in this thesis provided valuable information on T4 uptake by the intestine. Chapter 6 gives an overview of conditions of the intestinal tract, drugs and food decreasing the uptake of T4. Also the best timing of LT4 intake is discussed using the study results described in chapter 2 and 3. The importance of an acidic environment is stressed, with description of clinical conditions influencing the acidity of the stomach/ intestine. The data from the basal experiments in Caco2 cells are used to show the pH dependent transport of T4 in intestinal cells. New forms of T4 (capsules and oral liquid) are described that could be less dependent on pH and factors decreasing absorption by the intestine. In some patients TSH levels stay high despite high dose of LT4. Practical guidelines are given how to approach these patients. Factors decreasing LT4 absorption should be excluded, but if no reason for malabsorption can be found, pseudomalabsorption (noncompliance) should be considered. A guideline is given how to approach this problem.

CONCLUSION

In this thesis the treatment of hypothyroidism and absorption of T4 is described from a clinical and basic point of view. Put together the thesis gives insight in the factors influencing LT4 absorption and its results have influenced the timing of LT4 intake. The study of T4 uptake in Caco2 cells has given the first data on characteristics of intestinal T4 and T3 uptake. With these data further research can be performed, which will hopefully unravel the (different) transporter(s) involved in TH transport in intestinal cells.

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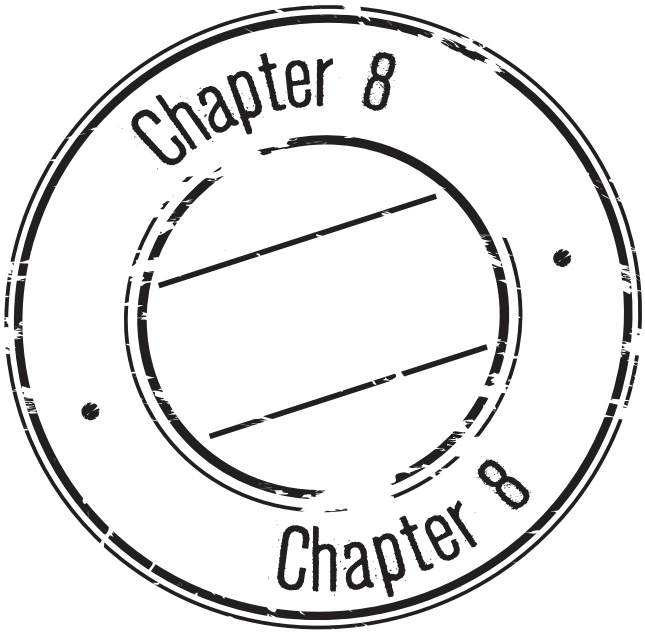


Summary

The normal thyroid gland produces the thyroid hormones, thyroxine (T4) and triiodothyronine (T3) under the influence of thyrotropin (TSH), produced by the pituitary gland. Thyroid hormone is essential for the regulation of energy metabolism in virtually all tissues. A lack of thyroid hormone, hypothyroidism, results in complaints of fatigue, weight gain, constipation and cold intolerance. Hypothyroidism is treated by replacing the lack of thyroid hormone by synthetic T4, levothyroxine. To guarantee the absorption of levothyroxine from the intestine, prescribing information advise intake of the tablet on an empty stomach, 30-60 minutes before breakfast. These instructions can be inconvenient for patients, and might influence the compliance. Furthermore, the reason for the reduced quality of life (QOL) in many treated hypothyroid patients has not been cleared up. This thesis describes the treatment of hypothyroidism and the absorption of (synthetic) T4 in the intestinal tract.

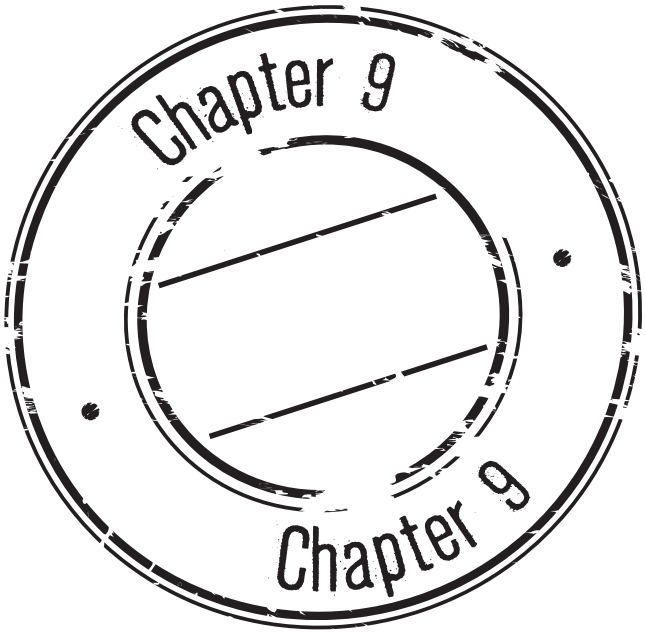
Chapter 1 provides the necessary background information on hypothyroidism and the treatment with levothyroxine. In the next chapters the performed studies are described

In the outpatient clinic we observed several patients that had an improvement in thyroid hormone levels and well being when they took levothyroxine at bedtime instead of in the morning. This prompted us to study the effect of switching levothyroxine intake from the morning to bedtime. As TSH has a circadian rhythm, we first studied the effect of bedtime levothyroxine on TSH and thyroid hormone profiles. **Chapter 2** describes a study in 11 hypothyroid patients treated with levothyroxine. A significant difference in TSH, T4 and T3 was found after switching to bedtime administration of levothyroxine. The circadian rhythm of TSH stayed intact. **Chapter 3** describes a randomized, double blind crossover trial in 90 patients on levothyroxine intake in the morning versus at bedtime. This trial confirmed the improved thyroid hormone levels with bedtime levothyroxine intake. Levothyroxine intake at bedtime is therefore a good alternative to morning levothyroxine intake. QOL parameters showed no significant changes, however. As the reduced QOL in hypothyroid patients is not understood, we performed a post-hoc analysis on the relation of QOL and the various parameters measured in the randomized trial. **Chapter 4** shows an inverse relationship between QOL and BMI in our cohort. Weight gain thus needs more attention

in the treatment of hypothyroidism. A relationship between QOL and thyroid hormone levels could not be found.

To clarify the mechanism of thyroid hormone uptake in the small intestine, we studied the transport of T4 and T3 in an intestinal cell model, the human colorectal adenocarcinoma cell line (Caco2). After reaching confluence, Caco2 cells differentiate spontaneously into enterocyte-like cells, and are therefore commonly used to study transport of various compounds in the small intestine. In **Chapter 5** the characteristics of T4 and T3 transport in Caco2 cells are described. Evidence is provided that amino acid transporters are important for uptake of T4 and T3 by Caco2 cells. T4 uptake is Na^+ independent and pH dependent, and inhibition studies suggest that it is mediated by the L-type amino acid transporter, LAT1. T3 uptake is Na^+ - and pH independent, and appears to be mediated by a T-type amino acid transporter, most likely MCT10. More experiments are required to identify the transporters responsible for thyoid hormone transport in the human intestine.

In the treatment of hypothyroid patients, an adequate absorption of levothyroxine by the intestine is essential. **Chapter 6** gives an overview of the available data on levothyroxine absorption and factors interfering with this absorption. Interference of levothyroxine uptake by gastrointestinal disorders, food and several drugs are described, and an advise is given on the best time of levothyroxine intake. When thyroid hormone levels do not return to normal, despite adequate levothyroxine treatment, malabsorption should be excluded.



Samenvatting

De schildklier ligt aan de voorzijde van de hals, voor het strottenhoofd, en maakt schildklierhormoon uit jodium en tyrosine. Zo ontstaat het inactieve hormoon thyroxine, T4. Als er een joodatoom van T4 wordt afgescheiden ontstaat het actieve hormoon, T3. De schildklier maakt met name T4 en ook een kleine hoeveelheid T3. Het grootste deel van het actieve T3 wordt omgezet vanuit T4 in de weefsels waar het op dat moment nodig is. De aanmaak van T4 wordt geregeld door de hypothalamus en de hypofyse in de hersenen. De hypothalamus stimuleert de hypofyse om schildklier stimulerend hormoon (thyroidstimulerend hormoon, TSH) af te scheiden, wat op zijn beurt de schildklier activeert om T4 te maken. Doordat T4 en T3 de hypothalamus en hypofyse weer remmen, wordt de hoeveelheid schildklierhormoon in het bloed binnen nauwe grenzen gehouden. Schildklierhormoon is belangrijk voor de stofwisseling in vrijwel alle weefsels in het lichaam. Een te traag werkende schildklier, hypothyreoidie, leidt tot een te trage stofwisseling. Hierbij ontstaan klachten zoals vermoeidheid, gewichtstoename, obstipatie en kouwelijkheid.

In de behandeling van hypothyreoïdie moet het tekort aan schildklierhormoon worden aangevuld op een manier die het meest de natuurlijke werking van schildklierhormoon nabootst. Vroeger werd hiervoor gedroogd dierlijk schildklierhormoon gebruikt. De hoeveelheid T4 en T3 was hierbij echter zeer variabel per medicijn. Sinds de 70-er jaren is nagemaakt (synthetisch) T4, genaamd levothyroxine, beschikbaar als behandeling. Hiermee kan een constante hoeveelheid T4 worden ingenomen, die in het lichaam wordt omgezet in actief T3. De huidige internationale richtlijnen schrijven levothyroxine voor als behandeling voor mensen met hypothyreoïdie. Hiermee kunnen de schildklierhormoonwaarden in het bloed weer worden hersteld. Om goede opname van levothyroxine in de darm te garanderen moet de tablet op een nuchtere maag worden ingenomen, omdat anders de opname verminderd kan worden door voedsel of andere medicijnen. De bijsluiter vermeldt daarom dat de tablet minstens een half uur voor het ontbijt moet worden ingenomen. Het kan lastig zijn voor patiënten om te wachten met ontbijt, wat de therapietrouw negatief kan beïnvloeden. Daarnaast blijven sommige patiënten klachten houden van hypothyreoïdie ondanks dat de schildklierhormoonwaarden in het bloed adequaat zijn. Dit proefschrift beschrijft de behandeling van hypothyreoïdie en de opname van levothyroxine door de darm. In

Hoofdstuk 1 wordt achtergrondinformatie over hypothyreoidie en de behandeling gegeven en worden de verrichtte studies ingeleid. In de volgende hoofdstukken worden de studies beschreven.

Op de polikliniek Interne Geneeskunde zagen wij enkele patiënten die zich beter voelden als ze de tablet levothyroxine 's avonds voor het slapengaan innamen, met hierbij ook verbeterde schildklierhormoon waarden. Dit was de reden om te onderzoeken of inname van levothyroxine rond bedtijd inderdaad een verbetering van schildklierwaarden geeft, en wat het effect op kwaliteit van leven is. Omdat TSH een dagritme heeft, met hogere waarden in de vroege nacht, werd eerst een studie verricht in een kleine groep patiënten om het effect van een ander inname tijdstip op het TSH dagritme te onderzoeken. Als het dagritme hierdoor verandert, moet hier namelijk rekening mee worden gehouden bij het tijdstip van bloedafname. In **Hoofdstuk 2** wordt de studie beschreven waarbij in in 11 patiënten die levothyroxine gebruikten, gedurende 24 uur elk uur bloed werd afgenomen. Na deze dag werd de patiënten gevraagd om de tablet levothyroxine 's avonds in te gaan nemen. Na 2 maanden werd opnieuw gedurende 24 uur bloed afgenomen. In deze kleine groep patiënten bleken de schildklierwaarden bij de avondinname al verbeterd, met een lager TSH en hogere T4 en T3 waarden. Het TSH dagritme bleef onveranderd met de avondinname van levothyroxine.

Om de resultaten van de kleine studie te bevestigen werd een grotere studie in 90 patiënten uitgevoerd die in **Hoofdstuk 3** wordt beschreven. Gedurende 6 maanden slikten patienten met hypothyreoidie 's ochtends en 's avonds een capsule waarvan de een levothyroxine bevatte en de andere een placebo. Na 3 maanden werden de capsules omgewisseld (cross over). Via loting (randomisatie) bij de apotheek, werd bepaald of een patiënt eerst levothyroxine in de ochtend of in de avond slikte. Zowel de patiënt als de dokter waren niet op de hoogte welke capsule de werkzame stof bevatte (dubbelblind). Elke 6 weken werden de patiënten teruggezien op de polikliniek en werd bloed geprikt en werden gewicht, hartslag en bloeddruk gemeten. Aan het begin, halverwege en aan het einde van de studie vulden de mensen 3 vragenlijsten in die de kwaliteit van leven meetten. Na afloop van de studie bleek inderdaad het TSH gehalte lager en de T4 en T3 waarden hoger bij avondinname van

levothyroxine. Dit wordt mogelijk verklaard door betere opname door de darm in de avond.

Ondanks de verbeterde schildklierhormoon waarden, was er geen verbetering in de kwaliteit van leven. In **Hoofdstuk 4** wordt de reden van de verminderde kwaliteit onderzocht door de verzamelde gegevens uit de gerandomiseerde studie te analyseren. Hierbij blijkt er geen relatie tussen schildklierhormoon waarden en kwaliteit van leven. Dit bevestigt de resultaten van eerdere studies. Wel bleek een hoger gewicht, en dus een hogere body mass index (BMI, kg/m^2) gerelateerd aan een slechtere kwaliteit van leven. Gewichtstoename behoeft daarom extra aandacht in de behandeling van patiënten met hypothyreoïdie. In de discussie van het hoofdstuk wordt ook ingegaan op andere mogelijke redenen van een verminderde kwaliteit van leven. Onder andere is er een mogelijkheid dat de hoeveelheid schildklierhormon in het bloed niet de hoeveelheid schildklierhormoon in de perifere weefsels weergeeft. Er is echter geen goede methode beschikbaar om de hoeveelheid schildklierhormoon in de weefsels makkelijk te meten. Ook zou het kunnen dat sommige patiënten waarbij de omzetting van T4 naar T3 minder goed verloopt, meer gebaat zijn bij een combinatie van T4 en T3 therapie. Meer onderzoek naar de beste individuele behandeling van hypothyreoïdie is noodzakelijk om de kwaliteit van leven van mensen met hypothyreoïdie te verbeteren.

Tijdens de studies naar avondinname van levothyroxine bleek dat het mechanisme van schildklier opname door de darm nog niet volledig was opgehelderd. Uit eerdere studies was wel gebleken dat de opname (transport) vooral plaats vindt in de twaalfvingerige darm en de dunne darm, echter hoe het schildklierhormoon de celwand passeert is niet duidelijk. In het laboratorium van de afdeling Interne Geneeskunde/ Endocrinologie van het Erasmus MC bleek het mogelijk om het transport van schildklierhormoon door de darmcel te bestuderen. De verrichtte experimenten worden beschreven in **Hoofdstuk 5.** Aangezien er geen menselijke dunne darmcellen beschikbaar zijn waarin transport kan worden bestudeerd, werden cellen van een dikke darm tumor (coloncarcinoom, Caco2) gebruikt die bij groei sterk lijken op de dunne darmcel. Om die reden wordt deze cellijn vaak gebruikt om transport in de dunne darm te bestuderen. Aanvankelijk werd gedacht dat schildklierhormoon de celwand gewoon kon passeren (diffusie).

Er is echter gebleken dat schildklierhormoon een transporter eiwit (soort celpoortje) nodig heeft om de celwand te passeren en de binnenkant van de cel te bereiken. Uit eerder onderzoek is gebleken dat schildklierhormoon transport in verschillende cel soorten in het lichaam gebeurt door verschillende transporter eiwitten. Deze verschillende transporters hebben allemaal karakteristieke eigenschappen die het transport van stoffen bevorderen of remmen. Om de karakteristieken van het schildklierhormoon transport in de darmcellen te achterhalen werden diverse experimenten uitgevoerd. Hieruit is gebleken dat transport van T4 en T3 waarschijnlijk gebeurt door verschillende transporters. Voor beide hormonen is het transport niet afhankelijk van een zout milieu (natrium). T4 transport is echter, in tegenstelling tot T3 transport, zeer afhankelijk van de zuurtegraad van de omgeving. Bij een zuur milieu (lage pH) is de opname van T4 veel beter. Vervolgens werden er bij de opname experimenten diverse stoffen toegevoegd waarvan bekend is dat zij bekende transporters kunnen blokkeren. Als de opname van T4 of T3 geremd wordt bij het toevoegen van een bepaalde stof, kan dit een sterke aanwijzing zijn dat de betreffende transporter betrokken is bij het schildklierhormoon transport. Hieruit kwam naar voren dat de transporter LAT1 een belangrijke rol lijkt te spelen bij de opname van T4 in de Caco2 cel. Voor de opname van T3 lijkt de transporter MCT10 van belang. De vraag blijft in hoeverre de opname van T4 en T3 in het cel model Caco2 een afspiegeling is van de werkelijke opname in de darmcel van de mens. Het is bekend dat de genoemde transporters aanwezig zijn in de dunne darmcel bij de mens. Het eiwit MCT10 lijkt zich echter aan de onderkant (basolaterale zijde) van de cel te bevinden, zodat het niet direct betrokken kan zijn bij de opname van T3. Mogelijk beïnvloedt MCT10 de uitwisseling van stoffen door een andere tranporter die zich wel aan de darmzijde (apicale zijde) van de cel bevindt. Waar de transporter LAT1 zich precies in de menselijke darmcel bevindt is nog niet duidelijk. Aanvullend onderzoek lijkt dus noodzakelijk om de exacte manier van schildklierhormoon opname door de darm op te helderen. In ieder geval hebben de verrichtte experimenten al veel meer inzicht gegeven over de mogelijke kandidaat transporters voor schildklierhormoon.

In **Hoofdstuk 6** wordt een overzicht gegeven van alle factoren die de opname van levothyroxine in de darm kunnen beïnvloeden. De verschillende darmaandoeningen, zoals glutenintolerantie (coeliakie),

maagontsteking (gastritis) en darmoperaties, die de opname van levothyroxine kunnen verminderen worden beschreven. Ook worden alle medicijnen en voedingsstoffen die de opname van levothyroxine beïnvloeden beschreven. Een advies wordt gegeven over het beste tijdstip om levothyroxine in te nemen. Ten slotte, worden adviezen gegeven voor het probleem waarbij het TSH gehalte hoog blijft, ondanks een hoge dosis levothyroxine. Als alle factoren die levothyroxine negatief beinvloeden zijn uitgesloten, duidt dit meestal op therapieontrouw.

Ten slotte wordt in Hoofdstuk 7 een overzicht gegeven van de verrichtte studies, en wordt besproken wat de invloed van de studies is op de dagelijkse praktijk. Verder worden suggesties gedaan voor verder onderzoek in de toekomst.

DANKWOORD

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PHD PORTFOLIO

Courses

2004 Auteurscursus Nederlands Tijdschrift van Geneeskunde
2005 Advanced Life Support
2005 Diabetes Education Study Group, Hoevelaken
2008 Module Ziekenhuismanagement
2000 Good Clinical Practice
2010 Teach the Teacher
2010 DESG Diabetes Update Course

Oral Presentations

2004 *'Effect of time of L-thyroxine administration on serum thyroid hormone profiles'*
Klinische Endocrinologie Dagen, Doorwerdt
2004 *'Effect of time of L-thyroxine administration on serum thyroid hormone profiles'*
Nederlandse Internistendagen, Maastricht
2005 *'Effect of time of L-thyroxine administration on serum thyroid hormone profiles'*
Wetenschapsavond Medisch centrum Rijnmond Zuid, Rotterdam
2007 *MEN X- syndrome?*
Endocrinologie van het Metabolisme, Utrecht
2009 *'Effects of evening versus morning levothyroxine: a randomized double-blind cross-over trial'* Annual Meeting Endocrine Society, Washington
2010 *'QOL in hypothyroidism related to BMI'*
International Thyroid congress, Paris, France
2010 *'Thyrotoxicosis in germ cell tumour'*
Dutch Serbian Meeting, Leiden
2011 *'Levothyroxine 's ochtends of 's avonds'*
Wetenschapsavond Rijnland Ziekenhuis, Leiderdorp

Poster presentations

2004 *'Effect of time of L-thyroxineadministration on serum thyroid hormone profiles'*
Annual Meeting Endocrine Society, New Orleans, USA
2010 *'Transport of T4 and T3 in intestinal (Caco2) cells'*
International Thyroid Congress, Paris, France

Conferences

2003 Annual Meeting European Thyroid Association, Edinburgh, Scotland
2003 Nederlandse Internistendagen, Maastricht
2004 Annual Meeting Endocrine Society, New Orleans, USA
2004 Nederlandse Internistendagen, Maastricht
2004 Erasmus Endocrinologie Cursus, Noordwijkerhout
2005 Nederlandse Internistendagen, Maastricht
2005 Erasmus Endocrinologie Cursus, Noordwijkerhout
2006 Nederlandse Internsitendagen, Maastricht
2006 Erasmus Endocrinologie Cursus, Noordwijkerhout
2007 Regionale Endocrinologie avond, Rotterdam
2007 Klinische Endocrinologie Dagen, Doorwerth
2007 Erfelijke stofwisselingsziekten van neonaat tot volwassene, Driebergen
2007 Endocrinologie van het Metabolisme, Utrecht
2007 Symposium Op het grensvlak van vasculaire geneeskunde en nefrologie, Rotterdam
2007 Annual Meeting Endocrine Society, Toronto, Canada
2007 Erasmus Endocrinologie Cursus, Noordwijkerhout
2007 Clinical Update Endocrinology, Manchester, UK
2008 Erasmus Endocrinologie Cursus, Noordwijkerhout
2008 Klinische Endocrinologie Dagen, Maarssen
2008 Endocrinologie van het Metabolisme, Utrecht
2009 Klinische Endocrinologie Dagen, Maarssen
2009 Nederlandse Internistendagen, Maastricht
2009 Endocrinologie van het Metabolisme, Utrecht
2010 Jaarsymposium Continuum Endocrinologie, Utrecht
2010 International Thyroid Congress, Paris, France
2010 Annual Meeting Endocrine Society, San Diego, USA
2012 International Congress of Endocrinology, Florence, Italy
2013 Jaarsymposium Continuum Endocrinologie, Utrecht
2013 Nederlandse Internistendagen, Maastricht
2013 Annual Meeting European Association for the Study of Diabetes, Barcelona, Spain
2015 Dutch Endocrine Meeting, Noordwijkerhout
2015 Clinical Endocrinology Update, Boston, USA
2015 Nederlandse Internistendagen, Maastricht

Teaching activities

2009	Lectures on thyroid (dys)function second year medical students
2010	Supervisor medical students and residents Internal Medicine Alrijne to Hospital, Leiderdorp
2015	Frequent education of students on subjects in Endocrinology
2012	Education of general practitioners on Osteoporosis and Diabetes
2015	Education of general practitioners on Endocrinology

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Kelderman-Bolk N., Berghout A., Visser T.J. (2015) Intestinal absorption of thyroxine. Review. *Submitted*

CURRICULUM VITAE

Nienke Bolk was born on December 30th 1974 in Groningen. She was raised in Leiden, where she graduated from the Stedelijk Gymnasium in 1993. Afterwards she took the Testamur in English Course in Exeter, United Kingdom. In 1994 she started her study at the Medical School of the Erasmus University in Rotterdam. As a medical student she spent an elective in Moshi, Tanzania. After graduating in 2001, she worked in the Zuiderziekenhuis (currently Maasstad Hospital) at the department of Internal Medicine. In 2003 she started her specialist training in General Internal Medicine at this hospital, under the supervision of Dr. A. Berghout. Together with him she started the research which lead to this thesis. She continued her training at the Erasmus Medical Centre Rotterdam (supervisor Prof. Dr. J.L.C.M. van Saase), and completed the subspeciality training in Endocrinology (supervisor Prof. Dr. A.J. van der Lelij). During this time she continued her research with dr A. Berghout and performed basic research in the group of Prof. dr. ir. T.J. Visser, which is described in this thesis. From 2010 she started her work as Endocrinologist in the Rijnland Hospital (currently Alrijne Hospital) in Leiderdorp. Nienke is married and has two children.