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Thyroid axis challenges in Leiden Longevity Study

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THYROID AXIS CHALLENGES IN LEIDEN LONGEVITY STUDY



ANA ŽUTINIĆ

Thyroid axis challenges in Leiden Longevity Study

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Ana Žutinić. Location: Wat Arun temple, Krung Thep Maha Nakhon, Thailand

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Thyroid axis challenges in Leiden Longevity Study

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Chapter 1

General introduction

Ana Žutinić

As the older population makes up a continuously increasing fraction of the whole demographic (1), understanding healthy ageing and deciphering the mechanisms underlying it, is now more valuable than ever. The sheer number of persons aged 60 years and older worldwide is expected to more than double by the year 2050, to an astounding 2.1 billion, from 962 million in 2017(2). Furthermore, the worldwide number of persons aged 80 years and older is expected to triple in this same period, to 425 million from 137 million in 2017(2). In the Netherlands, both the percentage of the population aged 65 and older, as well as the percentage of the population aged 80 years and older, are expected to greatly increase by the year 2050 compared to year 2015 (3), and with them the size of the population aged > 85 years, the ‘oldest old’.

Considering that age is the main risk factor for development of chronic diseases due to the time-dependent functional decline that occurs with it(4), detailed investigation of factors contributing to healthy ageing and of the mechanisms underlying the ageing process is not only significant for the individual but also crucial for sustainability of health care and the welfare of society, not only locally but globally. However, healthy ageing is a complex process.

Thyroid axis

One of the systems influencing healthy ageing might be the thyroid axis (figure 1). The thyroid axis is a complex network that regulates circulating levels of thyroid hormones, which in turn are major regulators of multiple physiological processes - from mood and cognition, to growth and development, to immunity and metabolism (5,6).

Feed forward and feedback regulation

The thyroid axis consists of several hormones which create a tightly regulated feed forward and feedback loop. Thyrotropin releasing hormone (TRH) is secreted by the hypothalamus and stimulates the secretion of thyroid stimulating hormone (TSH) from the anterior pituitary gland. TSH in turn stimulates the thyroid gland, through

TSH receptors (TSH-R), to produce and release thyroid hormones into the circulation. The circulating thyroid hormones (TH) inhibit the release of TRH and TSH through negative feedback, thereby maintaining an euthyroid state.

The thyroid gland itself is the place of thyroglobulin (Tg) production and storage and, from Tg, thyroid hormone production and secretion into the systemic circulation upon signalling from TSH. The thyroid gland produces mostly (90%) the prohormone thyroxine (T4) and partially also the active hormone, triiodothyronine (T3).

Circulating thyroid hormones are mostly bound by transport proteins (thyroglobulin binding protein (TBG), thyroxin-binding albumin (TBA), and albumin), although a small fraction is in free form (fT4 and fT3).

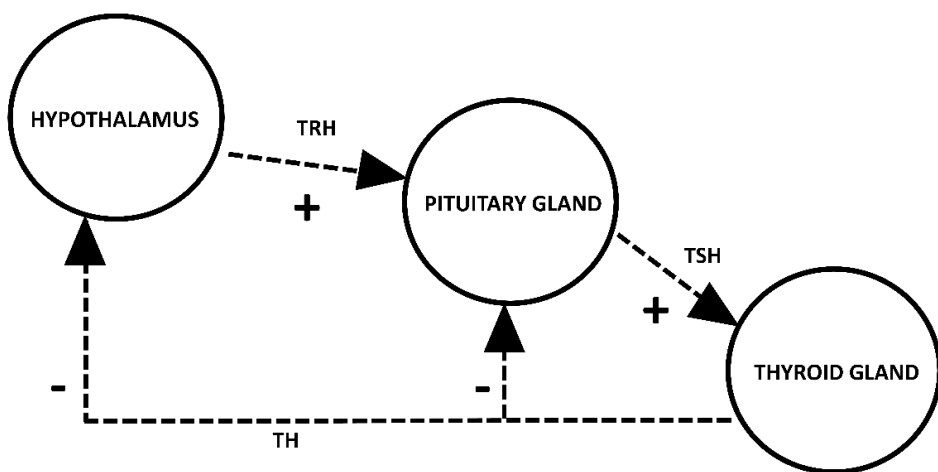


Figure 1. The negative feedback and feedforward system of the thyroid axis. TRH: thyrotropin releasing hormone, TSH: thyroid stimulating hormone, TH: thyroid hormones.

Tissue-specific regulation

Thyroid hormones can be taken up by cells from the circulation via specific transporter proteins. After cellular uptake, thyroid hormones can be activated and,

following binding to nuclear thyroid hormone receptor complexes, affect transcription. Deiodinase enzymes in tissues such as the liver, kidney, skeletal muscle and the thyroid itself, can activate the prohormone T4 into the active hormone T3 or deactivate the prohormone T4 into inactive reverse T3 (rT3) (7).

Pathophysiology

The influence of the thyroid axis is clearly apparent in its pathophysiology; the symptoms associated with hypo- (deficiency in circulating TH) and hyperthyroidism (surplus of circulating TH) are severe, devastating for the health and quality of life of the patient, and potentially fatal (5,6). Symptoms can range from weight gain, xerosis, hair loss, tiredness, dyslipidaemia, mood impairment and even myxoedema coma in the case of hypothyroidism; to weight loss, mood impairment, atrial fibrillation, muscle wasting and osteoporosis in hyperthyroidism (5,8).

Thyroid axis and ageing

Considering the vast implications of the thyroid axis in essential physiological processes, it is not surprising that it is also implicated in healthy ageing and longevity. Ageing is considered to be based on specific hallmarks: primary (genomic instability, telomere attrition, epigenetic alternations, loss of proteostasis), antagonistic (deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence) and integrative (stem cell exhaustion, altered intercellular communication)(4). The thyroid axis could influence multiple of these processes and thereby affect the rate of ageing. Indeed, various studies have shown a relationship between the thyroid axis and ageing and longevity (9-11).

In animal models, it has been shown that TH themselves may influence ageing adversely, as lower TH levels in various model organisms were associated with extended lifespan (10,12,13). The beneficial effects of low TH levels are attributed to lower basal metabolic rate and thereby lower levels of reactive oxygen species (ROS) and oxidative stress, the effects of which could lead to increased senescence (14). TH

can also adversely affect cell membranes as well as cell oxidant status, and even directly lead to increased cell senescence.

Moreover, the identification of TSH-R expression in tissues other than the thyroid, such as bone and adipose tissue (15,16), leaves possibility for TSH to exercise independent effects as well, not only through TH regulation but directly on tissues other than the thyroid gland.

In human studies, even within euthyroid range there is an association between lower thyroid status and ageing (11,17). Moreover, there is an increased prevalence of elevated TSH in the older population (18). Treating subclinical hypothyroidism (elevated circulating TSH in the absence of TH deficiency) in patients aged > 65 years has been shown through an extensive randomized controlled trial to have no apparent benefits (19) and other studies showed that familial longevity is actually associated with (genetic predisposition to) higher (normal) circulating TSH (9,20).

This thesis focuses on a subset of the participants from the Leiden Longevity Study (LLS, figure 2). The LLS was founded in 2002 specifically to study genotypes and phenotypes in healthy ageing (21). This unique cohort recruited long-lived siblings in the Netherlands without demographic restrictions other than age. Men, aged 89 and over, and women, aged 91 and over, respectively, were recruited in the study if they had another sibling of the same age criterium, thereby considering their family enriched for longevity. The offspring from this generation was also included in the cohort, as middle-aged subjects enriched for familial longevity, with their current partners as controls of similar age and with similar socio-economic background. In total, 421 families were included (22).

It has been confirmed that these long-lived siblings, as well as the offspring from these families, show familial (genetic and/or epigenetic) enrichment for extreme survival (21). Moreover, it was found that the offspring show less morbidity and mortality compared to controls (21).

Moreover, in LLS, a relationship was found between familial longevity and lower thyroid function (11) as well as low triiodothyronine levels (17). This does not seem to be the case in all familial longevity. In Ashkenazi centenarians and their offspring, another example of familial longevity, this relationship between the thyroid and longevity was not found (20). It could be that part of the LLS population has a lower thyroid setpoint, on which they thrive on lower TH levels and reap the benefit of longevity.

In 2015, the Switchbox consortium was established to investigate hormonal axes in longevity and healthy ageing. In a subgroup of 20 offspring and 18 controls from the LLS, a detailed observational study of thyroid hormone levels was performed - blood was sampled at high frequency (every 10 minutes) over a period of 24 hours and thyroid axis hormones measured at each time point. It was found that throughout the whole study period, offspring had on average a 0.8 mU/L higher circulating TSH levels than controls, despite an absence in differences in TH levels between the two groups (23). This unexpected finding sparked questions about the mechanism underlying this difference. The TSH bioactivity was not different between members of long-lived families and controls (23).

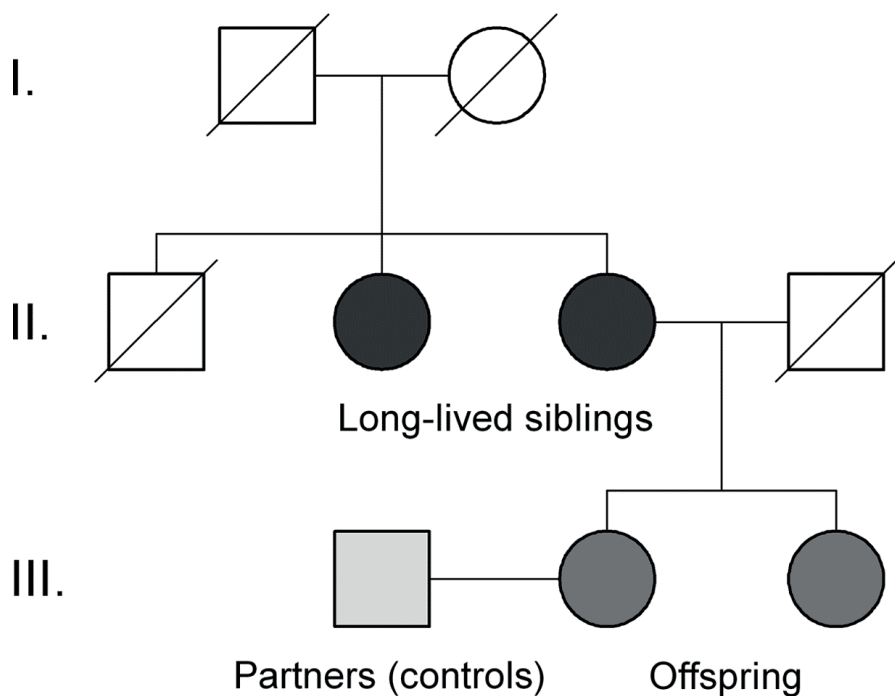


Figure 2. Leiden Longevity Study (LLS) – schematic representation of the design and family tree. Long-lived siblings (men aged 89 years or older, or women aged 91 years or older, with at least one sibling of the same age criterium) were considered enriched for familial longevity and were included in the LLS. They are descendants of the first generation. The offspring from these families are the third generation enriched for longevity, and their partners were included in the LLS as controls.

Hypothesis

In order to investigate the mechanism underlying the different thyroid axis status in members of long-lived families compared to controls, two hypotheses were formulated. In members of LLS where offspring had higher TSH than controls in the absence of differences in TH levels, we hypothesized that:

1) Offspring from long-lived families might have lower responsivity of the thyroid gland to stimulation by TSH, thereby needing higher levels for the same TH secretion,

and/or

2) Offspring from long-lived families might have higher turnover of TH in the circulation, thereby needing higher TSH levels in order to maintain adequate TH levels.

Objectives and rationale of this thesis

This thesis attempts to investigate the mechanism(s) underlying previously confirmed differences in thyroid status in offspring of long-lived families compared to controls in the Leiden Longevity Study.

The primary objective of these studies was to experimentally test the two hypotheses relating to TSH responsivity of the thyroid and TH turnover in members of long-lived families compared to controls. This was done by performing two thyroid axis challenge studies: one with recombinant human TSH (rhTSH, Thyrogen) and one with synthetic thyroid hormone triiodothyronine (T3) known as liothyronine (Cytomel).

The secondary objective of these studies was to explore secondary effects of these challenge studies on general physiology, tissue regeneration and the immune system through, during the challenge studies, obtaining data and performing analyses of: electrocardiography (ECG), metabolic parameters in blood and morning urine, markers of tissue regeneration (such as bone), immunological analysis through the collection of peripheral blood mononuclear cells (PBMCs), and biobanking of blood and urine samples for future analyses.

This thesis describes the first findings of two clinical challenge studies of the thyroid axis within the members of long-lived families and controls from the LLS. The layout is as follows: study design and data collection (Chapter 2), pilot study following the rhTSH challenge (Chapter 3), analysis of the primary outcomes in rhTSH challenge (Chapter 4), the primary outcomes in T3 challenge (Chapter 5) and first secondary outcomes - bone markers in rhTSH challenge study (Chapter 6). It is concluded with a general discussion placing the findings into context and providing future perspectives (Chapter 7).

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Chapter 2

Study design and data collection TSH and T3 study

Ana Žutinić

The THYRAGE (Resetting the THYROID axis for prevention of AGE-related diseases and co-morbidities) consortium consisted of six partners in five countries that started collaborations in January 2016 with the aim of investigating the role of the thyroid axis in resetting age related diseases. As part of this consortium, at Leiden University Medical Center (LUMC) two clinical challenge studies with thyroid drugs were performed in participants from the Leiden Longevity Study (LLS) in the period from March 2016 until September 2018.

Screening of participants

A subset of participants from the LLS(1), with a preference for subjects from the Switchbox study(2), who had TSH levels higher in offspring from long-lived families than in controls, were approached for screening through databases provided by the Department of Molecular Epidemiology, LUMC.

All interested subjects were globally screened by telephone (for history of cardiac, thyroid or endocrine disease) and upon passing this screening, were invited for a medical screening at the LUMC. The medical screening consisted of patient history, standard physical examination, a 10-second ECG recording in resting state and a single blood withdrawal. The following were measured at screening from fasted blood samples obtained and analysed by the Department of Clinical Chemistry and Laboratory medicine: sodium, potassium, creatinine, gamma GT, ASAT, ALAT, HbA1c, glucose, insulin, fT4, TSH, TPO antibodies, haematology (haemoglobin, haematocrit, erythrocytes, mean cellular volume, mean corpuscular haemoglobin (concentration), reticulocytes, leukocytes, erythroblasts). The inclusion and exclusion criteria were the same for both studies, and are noted in Table 1. Upon passing the medical screening, the participants had to participate in the study days within 8 weeks in order to minimise the risk of changes in health status between screening and study days.

Table 1. Inclusion and exclusion criteria for the TSH and T3 study.

Inclusion criteria	Member of Leiden Longevity Study, with higher TSH in offspring than in control as measured at baseline of Switchbox study.
Exclusion criteria	Cardiac arrhythmias (History) of thyroid diseases TSH level > 4.0 mU/l fT4 level outside of normal range (9-24 pmol/l) Any significant chronic disease Renal, hepatic or endocrine disease Hormone therapy Difficulties inserting an intravenous line Recent participation in other research projects (within the last 3 months), participation in 2 or more projects in one year Nicotine abuse or (History of) alcohol abuse (>28 units per week) Evaluation by the physician as too frail to participate

According to Dutch rules and regulations regarding research involving human subjects, the study protocol was submitted to two authorities, namely the local medical ethics committee (in April 2016) and the National Competent Authority (in May 2016) for approval. The Declaration of No Objection (DNO) from the National Competent Authority was received in May 2016 and the approval by the local medical ethics committee was granted in July 2016. The Board of Directors granted permission in July 2016 for performing the study at the LUMC. After these approvals, the inclusions of study participants could begin.

Between September 2016 and August 2018, 83 out of 135 participants in Switchbox were approached for participation in the TSH study, out of whom 30 were included in

the study (further details in Chapter 4). Participants who did not display the necessary TSH phenotype (higher TSH in offspring from long-lived families than in controls) were not approached. All TSH study participants were approached for the T3 study, however, only 25 could be included. An additional 8 participants from the original Switchbox pool were approached to try to reach 30 inclusions, however, only 2 could be included. The T3 study inclusion was completed at 27 participants (further details in Chapter 5). The timeline and number of inclusions for the TSH study and T3 study is shown in Figure 1 and Figure 2, respectively.

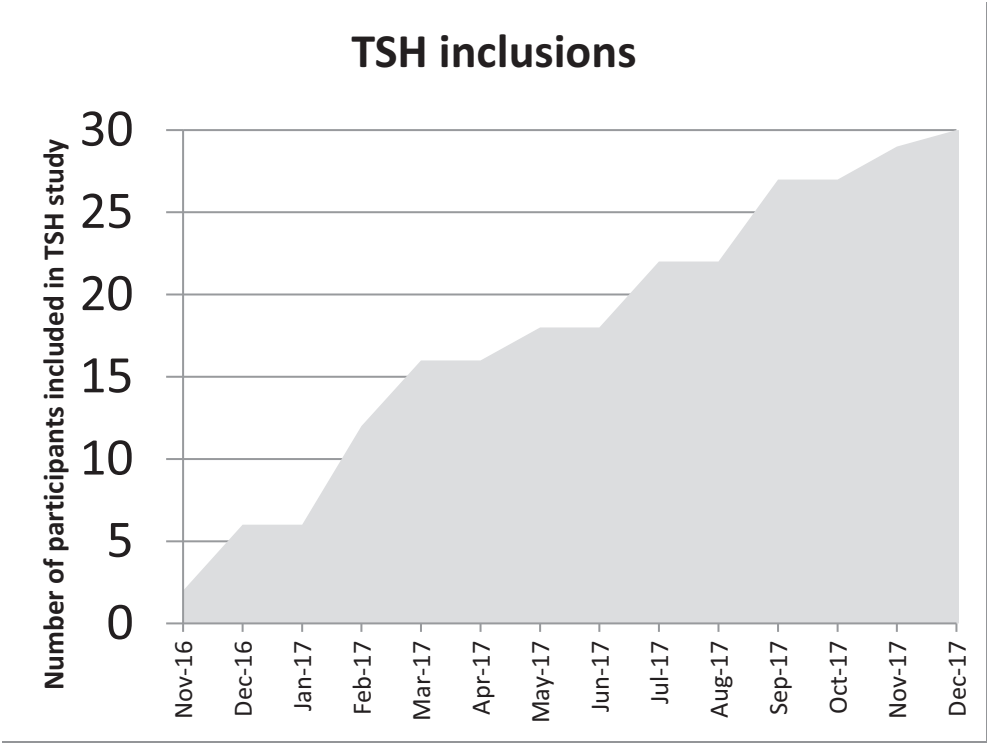


Figure 1. Number of participants and timeline of TSH study.

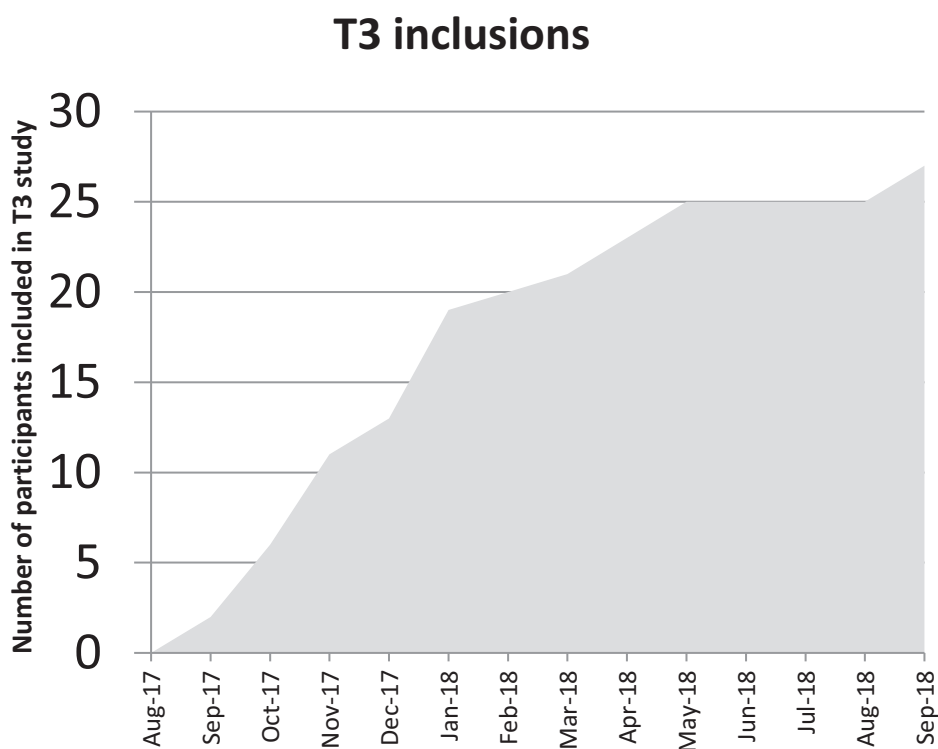


Figure 2. Number of participants and timeline of T3 study.

TSH study was always the first in order of participation. For participation in the T3 study, a minimum of three months since participation in the TSH study must have passed in order to allow adequate wash-out period of TSH study intervention and blood withdrawals on outcomes in the T3 study. Within each study, order of participation was at random, except for couples participating together. Per week, one to three subjects participated in the study, due to logistical reasons. The first TSH challenge study took place in November 2016, the last in December 2017. The first T3 challenge study took place in September 2017 and the last in September 2018.

Data collection TSH and T3 study

Blood sampling

At both studies, samples of serum, EDTA, PAX gene and whole blood samples were collected. In the TSH study, 255.5 ml of blood was withdrawn per participant, divided across 17 time points. In the T3 study, 370.0 ml of blood was withdrawn per participant, divided across 29 time points. The type of blood sample obtained, the volume of the blood and the frequency of withdrawal in the TSH challenge study are depicted in Table 2, and in the T3 study are depicted in Table 3.

Table 2. Overview of the blood sample type and frequency of sampling in the rhTSH challenge.

Measurement time	Number & type of tubes	Quantity of blood / tube (ml)	Total volume (ml)	Type of biomaterial	Measured parameters
14x day 1 + 1x day 2/3/4 (24, 48, 72h after injection)	2x serum 1x EDTA	3.5 2.0	17x 7.0 17x 2.0	Serum Plasma	Thyroid function + tissue turnover Markers tissue turnover
6x day 1 + 1x day 2/3/4 (24, 48, 72h after injection)	1x PAX gene 1x EDTA	2.5 4.0	9x 2.5 9x 4.0	RNA Plasma	Gene expression Metabolome
1x day 1 + 1x day 3 (72h after injection)	2x whole blood 1x EDTA	8.0 2.0	2x 16.0 2x 2.0	PBMCs Plasma	Immunophenotype
1x baseline + 1x day 3 (72h after injection)	1x EDTA	4.0	2x4.0	Plasma	Haematology

Table 3. Overview of the type of blood samples and frequency of blood sampling in the T3 challenge.

Measurement time	Number & type of tubes	Quantity of blood / tube (ml)	Total volume (ml)	Type of biomaterial	Measured parameters
25x day 1 + 1x day 2/3/4/5 (24, 48, 72, 96h after tablets)	2x serum 1x EDTA	3.5 2.0	29x 7.0 29x 2.0	Serum Plasma	Thyroid function + tissue turnover Tissue turnover
6x day 1 + 1x day 2/3/4/5 (24, 48, 72, 96h after tablets)	1x PAX gene 1x EDTA	2.5 4.0	10x 2.5 10x 4.0	RNA Plasma	Gene expression Metabolome
1x day 1 + 1x day 5 (96h after tablets)	2x whole blood 1x EDTA	8.0 2.0	2x 16.0 2x 2.0	PBMCs Plasma	Immunophenotype
1x baseline + 1x day 5 (96h after tablets)	1x EDTA	4.0	2x4.0	Plasma	Haematology

Urine sampling

Participants collected second void morning urine each morning of the study, on day 1,2,3,4 during rhTSH study and day 1,2,3,4,5 during T3.

Body composition measurements

On study day 2, participant's weight and height was measured and body composition parameters such as fat mass and lean mass were measured using a Bioelectrical Impedance Analysis meter at a fixed frequency of 50kHz (Bodystat 1500 Ltd, Isle of Man, British Isles (3)).

Continuous physiological measurements

Participants wore an Equivital monitor, which records continuous electrocardiography, accelerometry and breathing rate, and also records body temperature when used in combination with daily intake of a core body temperature capsule (Vivosense core body temperature capsule). The Equivital monitors used were Equivital EQ02 SEM from Hidalgo, UK, in combination with Equivital carry on belts, sizes 1 thru 9. On the morning of study day 1, the size of band was selected per participant, the Equivital monitor was connected to the band and participants started their log of Equivital wearing times. The participants were instructed to charge the Equivital monitor twice daily, in the morning and in the evening, until the battery light turned green. The times of not wearing the belt and charging were logged by the participants every day and checked by the study physician on each study day.

Questionnaires

Participants answered a basic questionnaire concerning age of their parents, dietary habits (the details of following a specific eating regimen) and regular exercise routine (per type of sport and weekly duration and frequency). This questionnaire was conducted by the same study physician in all participants.

Diaries

Participants kept a diary during study participation where they could log their exercise times, sleeping times, Equivital monitor charging times and any special remarks concerning meals. They received daily reminders during the study day to keep up their diary entries and were presented with the opportunity to ask any questions in case something was unclear.

Biobank sampling

All participants provided consent for storing biobank material. Samples for the biobank were withdrawn at each blood withdrawal time point. They are stored in accordance with the rules and regulations of the LUMC Biobank Ageing. The biobank samples are stored at the Department of Clinical Chemistry and Laboratory medicine (KCL) at the Leiden University Medical Center (LUMC), The Netherlands. This department is fully accredited (EN ISO 15189:2012) by the Dutch Accreditation Council.

Data management

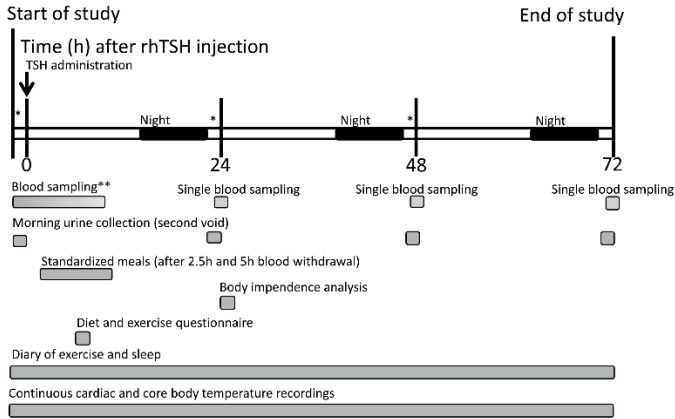
A secure and custom ProMISe database was created for this study. Information from paper CRFs was input by the study physician directly after the participants completed the study. Laboratory outcomes were imported from Excel files provided by the KCL.

Overview

A schematic representation of the study design and data collection in the TSH study and T3 study is provided in Figure 3A and B, respectively.

A

Schematic representation of study protocol TSH study

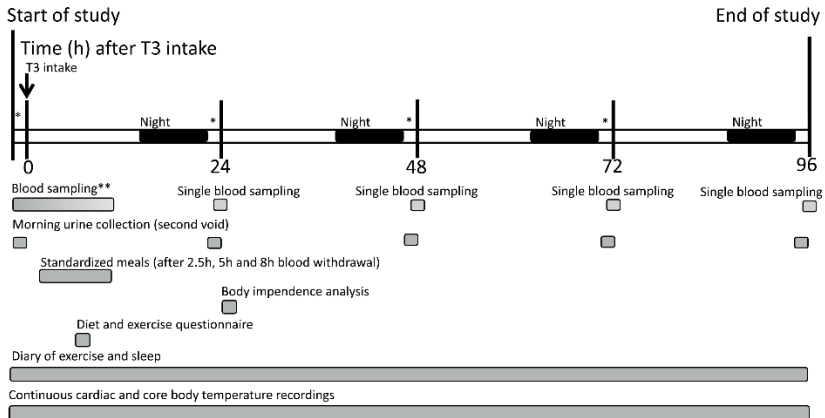


* Core body temperature new capsule intake

**Blood sampling frequency during study day 1: baseline before time 0, 0-1h every 15 min, 1-3h every 30 min, 3-8h every hour

B

Schematic representation of study protocol T3 study



* Core body temperature new capsule intake

**Blood sampling frequency during study day 1: baseline before time 0, 0-4h every 15 min, 4-7h every 30 min, 7-9h every hour

Figure 3. Schematic overview of the TSH study (A) and T3 study (B). TSH: thyroid stimulating hormone; T3: triiodothyronine.

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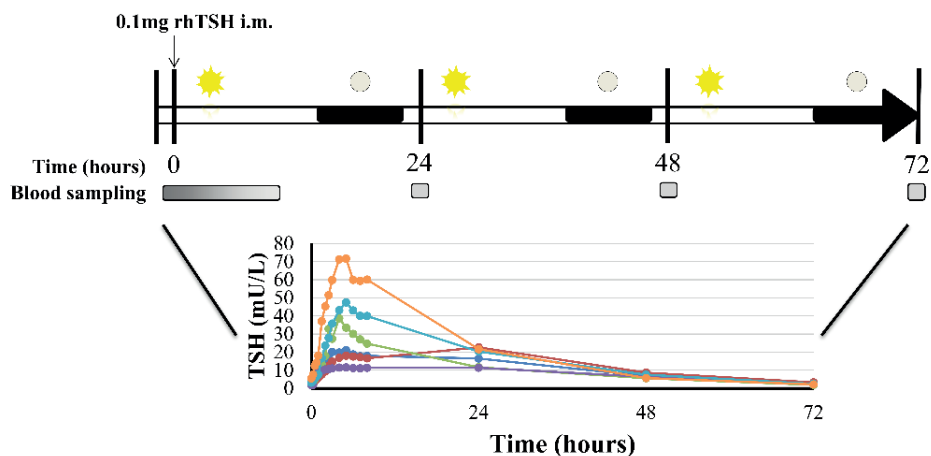
Chapter 3

Determining the Frequency of Thyroid Parameter Measurements Following rhTSH Administration in a Healthy, Older population. Ana Žutinić, Gerard J. Blauw and Diana van Heemst. (doi: 10.1016/j.mex.2021.101400, MethodsX 2021)

Abstract

Serial thyroid hormone measurement in blood following recombinant human thyroid stimulating hormone (rhTSH) administration has not been studied extensively in healthy, older populations. Current methods involve measurement of thyroid hormones mostly at 4 to 24 hours following rhTSH administration. We tailored existing protocols to measure thyroid hormones at high frequencies following 0.1mg rhTSH intramuscular (i.m.) administration to identify optimal measurement points in our healthy, older population. We designed a method with frequent blood sampling in the first 8 hours, followed by blood sampling at 24, 48 and 72 hours after rhTSH administration to measure TSH, thyroxine (T4), free T4 (fT4), triiodothyronine (T3), free T3 (fT3) and thyroglobulin (Tg). In total, we performed a series of 17 blood withdrawals in four consecutive days. Following 0.1mg rhTSH (i.m.) administration, mean thyroid parameters showed great inter-individual variation and variation over time. Mean TSH concentration showed the greatest variation in the first 8 hours following rhTSH administration. Mean T4, fT4, T3 and fT3 started showing variation from 2 hours after rhTSH administration, and were less variable than mean TSH concentration. Mean Tg was only variable at later time points, namely 24, 48 and 72 hours after rhTSH administration. In this novel method with high frequency blood sampling following 0.1mg rhTSH (i.m.) administration, we identify optimal time points for measuring thyroid gland output in a healthy, older population. Our methods and findings may be informative for further thyroid but also other hormonal axis studies.

Graphical Abstract



Background

Thyroid stimulating hormone (TSH) is the main driver of thyroid hormone production by the thyroid gland. Before characterization of the TSH gene, bovine as well as human cadaver TSH have been isolated for use in the clinic for detecting residual disease in patients with thyroid cancer(1). Following the characterization of the TSH subunits (2,3), recombinant human TSH (rhTSH) could be produced, in Chinese hamster ovary cells(4), and since then rhTSH has been used in the clinic as well as in preclinical studies comprising healthy young and middle-aged adult populations. Current literature provides limited information on the magnitude and variation in effects of rhTSH on the output of the thyroid gland in a healthy, older population.

In the Leiden Longevity Study (LLS), we recently showed that offspring from long-lived families have a different thyroid axis status than controls, albeit in the normal range, with offspring displaying higher circulating TSH levels than controls in the absence of differences in levels of thyroid hormones over a 24-hour period as measured every 10 minutes(5). Based on this finding, the hypothesis was formulated that the thyroid gland in offspring of long-lived families is less responsive to TSH

stimulation than the thyroid gland of controls. To test this hypothesis, we aimed to perform a thyroid axis challenge study with rhTSH in a healthy, older population.

Previous literature studying rhTSH dynamics reported effects on young and middle-aged adults, mostly measured at 24 hour intervals(6-8), although some studies reported thyroid parameter concentrations at 2 and 4 hours following rhTSH administration (9,10). However, since there is great variation in the magnitude of changes in thyroid parameters following rhTSH administration(7,10) and since there were indications that age modifies the response to rhTSH (7,11), uncertainty remained about the appropriate frequency of blood sampling for thyroid parameter measurements in our healthy, older population.

We performed a series of frequent blood sampling and measured thyroid parameters TSH, thyroxine (T4), free T4 (fT4), triiodothyronine (T3), free T3 (fT3) and thyroglobulin (Tg) in a small group comprising 6 participants, in order to determine the necessary frequency of measurement in the whole cohort. Here we report the method used to identify optimal time points for measurement of thyroid hormone parameters following intramuscular administration of 0.1mg rhTSH in a healthy, older population.

Method details

Study population

Study participants were recruited from LLS (12) , underwent a medical screening and were excluded based on criteria outlined in detail elsewhere (13). All participants gave written informed consent and the study was performed in accordance with the declaration of Helsinki.

Blood sampling frequency

On the morning of study day 1, an intravenous cannula was placed in a forearm vein, blood was withdrawn at baseline and rhTSH was administered through intramuscular injection (0.1 mg/mL in 1 mL, gluteal muscle). The time of injection was used as

reference, time zero. Blood was sampled at a high frequency following injection for optimal detection of circulating parameters reflecting the thyroidal response to rhTSH. In the first hour after injection, blood was sampled every 15 min. Between 1 and 3 h after injection, blood was sampled every 30 min, and finally between 3 and 8 h after injection, every hour. During study day 1, subjects received two standardized meals (two hours and five hours after rhTSH injection), each consisting of 600 kcal (2x125mL Nutridrink Compact, Nutricia Advanced Medical Nutrition, Zoetermeer, The Netherlands). On study day 2, 3 and 4, additional fasted blood samples were obtained at respectively 24, 48 and 72 h after rhTSH injection.

This is the highest frequency of sampling reported in literature following a rhTSH challenge.

Serum samples and laboratory measurements

Serum samples were kept at room temperature for 60 min to clot before processing at the Department of Clinical Chemistry and Laboratory Medicine, Leiden University Medical Centre, The Netherlands. Samples were centrifuged for 10 min at 2350 G relative centrifugal force at a temperature of 20 degrees Celsius. After being transferred to 500 microliter aliquots, serum samples were stored at –20 degrees Celsius prior to permanent storage at –80 degrees Celsius until analysis.

Laboratory measurements were performed after all subjects had completed the study. All measurements were performed with the same lot number. For each participant, samples from the different time points were measured in the same batch. Assays and assay performance are reported in detail elsewhere (13). In short, all measurements were performed with fully automated, software monitored equipment and diagnostics from Roche Diagnostics (Almere, The Netherlands) at the Department of Clinical Chemistry and Laboratory Medicine at Leiden University Medical Centre, The Netherlands. Thyroid parameters TSH (Catalogue number 11731459122, research reference identifier (RRID): AB_2756377), fT4 (Catalogue number 6437281190, RRID: AB_2801661), T4 (Catalogue number 12017709122, RRID: AB_2756378), fT3

(Catalogue number 6437206190, RRID: AB_2827368) and T3 (Catalogue number 11731360122, RRID: AB_2827369) were measured in serum by an immunoassay using Roche cobas8000 with an E602 module.

Determining thyroid parameter measurement frequency for whole cohort

Following completion of the study by all participants, samples from three female and three male (total n=6) participants who participated in both the rhTSH study as well as in a second study with T3, as a couple and for whom blood collection was successful at all time points, were used to measure thyroid parameters at all 17 time points in order to identify optimal measurement points for the whole cohort comprising 30 participants.

Results

The baseline characteristics of the pilot study participants are shown in Table 1, showing that study participants were of high-middle age and there was an equal distribution of men and women.

Thyroid parameters at baseline and following 0.1 mg rhTSH (i.m.) administration are shown in Table 2. Participants were euthyroid at baseline. Following 0.1 mg rhTSH administration, peak values of thyroid parameters were reached, although at different time points depending on the parameter. Circulating TSH, T3 and fT3 reached peak concentrations before 24 hours (namely at 5, 7.5 and 7h, respectively) following rhTSH administration. Peak circulating fT4 and T4 was reached at 48 h following rhTSH administration, while Tg concentration peak was reached at 72 hours following rhTSH administration. The greatest increase in concentration from baseline following rhTSH administration was seen in circulating TSH, followed by circulating Tg (1013% and 217%, respectively).

Table 1. Baseline characteristics of the study participants (n=6).

	All participants	Males	Females
Number	6	3	3
Age <i>years</i>	68 (2)	69 (1)	67 (2)
Weight <i>kg</i>	79.2 (19.9)	85.9 (12.8)	72.6 (26.4)
Height <i>cm</i>	170.5 (10.2)	178.8 (5.55)	162.1 (4.6)
BMI <i>kg/m²</i>	27.1 (5.8)	26.9 (3.6)	27.2 (8.5)
GFR <i>ml/min/1.73m²</i>	72.8 (9.8)	70.3 (9.1)	75.3 (11.7)
AST <i>U/L</i>	20.2 (5.1)	20.1 (6.1)	20.2 (5.4)
ALT <i>U/L</i>	19.1 (7.2)	23.7 (6.4)	14.5 (5.2)

All values are shown as mean (standard deviation). BMI: body mass index; GFR: glomerular filtration rate; AST: aspartate transaminase; ALT: alanine transaminase.

Table 2. Thyroid parameters at baseline and following 0.1 mg i.m. rhTSH administration in all study participants (n=6).

	Baseline	Peak	Time of peak (hours)	% increase from baseline to peak
TSH <i>mU/L</i>	2.9 (1.7-5.6)	30.7 (11.5-71.2)	5 (4-24)	1013 (331-1414)
fT4 <i>pmol/L</i>	15.3 (12.2-20.6)	20.9 (17.4-45.2)	24 (24-48)	42 (31-118)
T4 <i>mmol/L</i>	85.5 (84.7-110.6)	113.3 (100.2-183.1)	24 (24-48)	32 (26-65)
fT3 <i>pmol/L</i>	4.9 (4.4-5.3)	8.5 (7.1-14.5)	7 (5-24)	69 (59-174)
T3 <i>mmol/L</i>	1.6 (1.3-1.8)	2.7 (2.1-3.6)	7.5 (4-24)	63 (50-110)
Tg <i>µg/L</i>	11.6 (3.2-56.3)	46.6 (8.3-194.9)	48 (24-72)	217 (142-465)

All values are shown as median (minimum - maximum). rhTSH: recombinant human thyroid stimulating hormone; i.m.: intramuscular. TSH: thyroid stimulating hormone; fT4: free T4; T4: thyroxine; fT3: free T3; T3: triiodothyronine; Tg: thyroglobulin.

The peak times and intervals of the whole cohort (n=30) are also presented in Table 2 for comparison with our study (n=6). In the whole cohort, the peak times and intervals of TSH, T4, fT4 and Tg were comparable to our study population, further strengthening our results. T3 and fT3 in the whole cohort had comparable minimum but slightly later peak and maximum times compared to our study, further supporting

the necessity of multiple measurement time points due to large variation in a healthy, older population.

Mean (SD) thyroid parameters at baseline and following 0.1 mg rhTSH (i.m.) administration are shown in Figure 1. Eye-level evidence suggests that during the first 8 hours following rhTSH administration, circulating TSH concentration was the most variable while Tg concentration had very low variability.

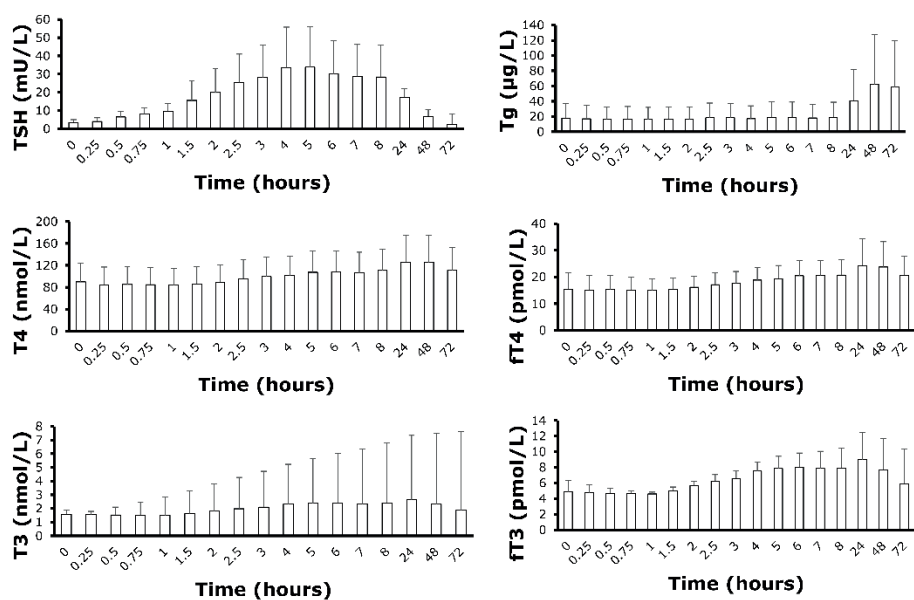


Figure 1. Mean thyroid parameters at baseline and at 16 timepoints divided across four consecutive days following 0.1mg rhTSH (i.m.) administration in a healthy, older population (n=6). Error bars represent standard deviation. rhTSH: recombinant human thyroid stimulating hormone; i.m.: intramuscular.

In fact, circulating TSH displayed the greatest variation from baseline during the first 8 hours following administration, and values started returning to baseline 24, 48 and

72 hours following rhTSH administration. FT4 and T4 had similar variability during the first 8 hours following rhTSH administration, as did fT3 and T3.

Thyroid parameters per participant (n=6) at baseline and following 0.1 mg rhTSH (i.m.) administration are shown in Figure 2. Eye evidence suggests considerable inter-individual variation in circulating thyroid parameters following rhTSH administration.

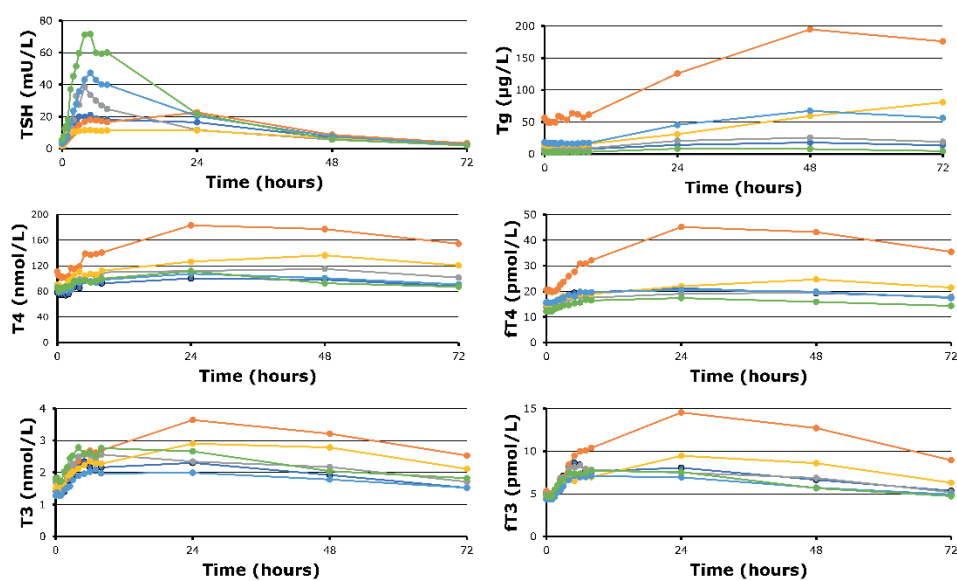


Figure 2. Thyroid parameters per participant in a healthy, older population (n=6) at baseline and throughout the study at 16 timepoints following 0.1mg rhTSH (i.m.) administration. rhTSH: recombinant human thyroid stimulating hormone; i.m.: intramuscular.

Considering the inter-individual variation but also great variation over time of thyroid parameters following administration of 0.1mg rhTSH, we recommend frequent measurement of TSH (every 30-60 minutes), followed by slightly less frequent measurements of fT4, fT3, T3 and T4 (every 1-2 hours) during the first 8 hours

following administration. Tg displayed very low responses during the first 8 hours following administration and may be measured at 24 hour intervals following administration.

Conclusion

In this novel study of thyroid parameter measurement frequency following 0.1mg rhTSH (i.m.) administration in a healthy, older population, we show detailed profiles of thyroid parameters and identify optimal measurement points for recording thyroid hormone responses to rhTSH administration. Our recommendation is to, following 0.1 mg rhTSH (i.m.) administration, measure TSH concentrations at a maximum frequency of every 15 minutes in the first 8 hours and to measure fT4, T4, fT3 and T3 concentrations (or alternatively, fT4 and fT3 concentrations only, since their trajectories closely resembled those of T4 and T3) at a frequency of 2 hours in the 8 hours following administration, followed by measurements at 24, 48 and 72 hours following administration. Tg displayed very low variability in the first 8 hours, and can therefore be measured at baseline, 24, 48 and 72 hours, following rhTSH administration. In future research, these findings can be used to optimally estimate frequency of blood withdrawals and optimize protocols for blood sampling frequencies in thyroid hormone and other hormone studies, especially in older populations where literature remains scarce.

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Conflict of interest

The authors have no conflict of interest.

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Chapter 4

Familial Longevity is Associated with an Attenuated Thyroidal Response to Recombinant Human Thyroid Stimulating Hormone. Ana Žutinić, Hanno Pijl, Bart E. Ballieux, Ferdinand Roelfsema, Rudi G.J. Westendorp, Gerard J. Blauw and Diana van Heemst. (doi: 10.1210/clinem/dgaa195, Journal of Clinical Endocrinology and Metabolism, 2020)

Abstract

CONTEXT. Longevity is associated with higher circulating levels of thyroid stimulating hormone (TSH) in the absence of differences in circulating thyroid hormones (TH), as previously observed in F2 members of long-lived families (F2-LLS) and their partners (F2-Con). The mechanism underlying this observed difference remains unknown.

OBJECTIVE. We hypothesized that the thyroid gland of members from long-lived families are less responsive to TSH stimulation, thereby requiring higher circulating TSH levels to maintain adequate TH levels.

METHODS. We performed a case-control intervention study with a single intramuscular (gluteal) injection with 0.1mg recombinant human TSH (rhTSH) in a subgroup of 14 F2-LLS and 15 similarly aged F2-Con. They were followed-up for 4 days. No serious adverse events were reported. For analyses, we compared time trajectories of TSH and TH, and the ratio of TH to TSH using area under the curve (AUC) calculations.

RESULTS. The AUC fT₄/AUC TSH ratio was significantly lower in F2-LLS than in F2-Con (estimated mean (95%CI) 1.6 (1.2-1.9) and 2.2 (1.9-2.6), respectively, $p=0.01$). The AUC Tg/AUC TSH ratio was also lower in F2-LLS than in F2-Con (median (IQR) 2.1 (1.4-3.6) and 3.2 (2.7-7.4), respectively, $p=0.04$). We observed the same trend with the AUC fT₃/AUC TSH ratio, although the difference was not statistically significant (estimated mean (95%CI) 0.6 (0.4-0.7) and 0.7 (0.6-0.8), respectively, $p=0.07$).

CONCLUSIONS. The present findings show that members of long-living families have a lower thyroid responsivity to TSH compared to their partners.

Precis

The results from this challenge study with 0.1mg recombinant human TSH in 29 participants indicate that the thyroid gland of members from long-lived families is less responsive to stimulation by TSH than the thyroid gland of similarly aged controls.

Introduction

Thyroid status changes with age(1) and plays an important role in multiple physiological processes. Circulating levels of thyroid hormones are tightly regulated through an interplay of feedforward and feedback mechanisms. Hypothalamic TRH stimulates the secretion of TSH from the pituitary which stimulates the thyroid gland to produce and release thyroid hormones into the circulation. Increases in circulating levels of thyroid hormones are centrally monitored and lead to inhibition of the release of TRH and TSH, which puts a halt to further increases in thyroid hormone production. With ageing, several changes occur in circulating parameters of the thyroid axis. Most strikingly, TSH levels tend to increase with age, a trend that was observed to extend into advanced ages(2), and which might be explained by selective survival of people with a genetic or familial predisposition for relatively higher TSH (3,4).

In line with these earlier findings, we observed that in advanced middle-age, in a sub study from the Dutch Leiden Longevity Study (LLS) comprising 38 participants from whom blood samples were taken every 10 min during 24 hours (h), that members from long-lived families (F2-LLS) had on average an 0.8mU/L higher serum concentration of TSH than the similarly aged reference group (their partners, F2-Con), while thyroid hormone levels were comparable between groups (5).

Additionally, in the same cohort, we observed a stronger temporal relationship between TSH and free T3 in F2-LLS than in F2-Con, but no differences in the feedback and forward interplay between TSH and thyroid hormones(6). The bioactivity of TSH has been shown to not differ between F2-LLS and F2-Con(5).

The aim of the present study is to test the hypothesis that the thyroid gland of F2-LLS is more resistant to stimulation with TSH compared to the thyroid gland of their partners, F2-Con. To investigate this, we recruited F2-LLS and F2-Con from a subgroup of LLS for a challenge study with a single dose of recombinant human TSH (rhTSH, Thyrogen®, Genzyme Corp., Framingham, MA), where we hypothesized that upon administration of the same low dose of recombinant human TSH, F2-LLS will have a lower thyroïdal response than F2-Con.

Materials and Methods

Study population

The Leiden Longevity Study (LLS) was founded in 2002 and designed to investigate genotypes and phenotypes underlying inter-individual differences in familial longevity in humans(7). In the LLS, family members of two different generations were included, comprising an F1 generation of long-lived siblings from 421 Caucasian long-living families (men aged 89 and older, women aged 91 and older) living in The Netherlands in early 2000s, without any restrictions on health or demographics (8). The offspring of these long-lived F1 siblings were also asked to participate in the study, with the offspring's partners as controls, thereby creating a case group enriched for longevity (F2-LLS) and a control group with similar lifestyle factors and socio-economic status, but without selection for familial predisposition to longevity (F2-Con).

Subjects were recruited for the TSH challenge study from the subgroup of LLS previously studied in terms of thyroïdal status between F2-LLS and F2-Con(5), and excluded based on the exclusion criteria mentioned below. The exclusion criteria were: laboratory results (haemoglobin < 7.1 mmol/L, TSH > 4.0 mU/L, fT4 < 9 pmol/L or > 24 pmol/L, TPO antibody positivity (>35 kU/L)), medical history (cardiac arrhythmias, (history of) thyroid diseases, renal, hepatic or endocrine disease, or any other significant chronic disease), medication use (hormone therapy, thyroid medication), lifestyle factors (nicotine abuse, (history of) alcohol abuse (>28 units per

week)) and other factors (difficulty inserting an intravenous cannula, participation in other research projects within the last 3 months, participation in two or more projects in one year, evaluation by a physician as too frail or vulnerable to participate).

Clinical protocol

Participants were admitted into the study after passing medical screening. The TSH challenge study consisted of four consecutive study days at Leiden University Medical Centre. On the morning of study day 1, an intravenous cannula was placed in a forearm vein, blood was withdrawn at baseline and rhTSH was administered through intramuscular injection (0.1 mg/mL in 1 mL, gluteal muscle). The time of injection was used as reference, time zero. Blood was sampled at a high frequency following injection for optimal detection of circulating parameters reflecting the thyroidal response to rhTSH. In the first hour after injection, blood was sampled every 15 min. Between 1 and 3 h after injection, blood was sampled every 30 min, and finally between 3 and 8 h after injection, every hour. During study day 1, subjects received two standardized meals (two hours and five hours after rhTSH injection), each consisting of 600 kcal (2x125mL Nutridrink Compact, Nutricia Advanced Medical Nutrition, Zoetermeer, The Netherlands). On study day 2, 3 and 4, additional blood samples were obtained at respectively 24, 48 and 72 h after rhTSH injection. Outside of these times, subjects were at their leisure.

The blood samples obtained at baseline, 15 min thru 2 h, and 24, 48 and 72 h after injection were drawn when participants were in the fasted state.

In total, 255.5 mL of blood was withdrawn from each subject across 17 time points (14 on study day 1, and 1 each on day 2, 3 and 4).

Height, weight and body composition were measured on study day 2. Body composition was measured with a Bioelectrical Impedance Analysis meter at a fixed frequency of 50kHz (Bodystat 1500 Ltd, Isle of Man, British Isles(9)).

The study was designed in accordance with the declaration of Helsinki and has been approved by the Medical Ethical Committee of the Leiden University Medical Centre. It is registered at Leiden University Medical Centre under the protocol P16.107 and with EudraCT under the number 2016-001497-15. All subjects gave written informed consent prior to the screening visit.

Handling of samples

Serum samples were kept at room temperature for 60 min to clot before processing at the Department of Clinical Chemistry and Laboratory Medicine, Leiden University Medical Centre, The Netherlands. Samples were centrifuged for 10 min at 2350 G relative centrifugal force at a temperature of 20 degrees Celsius. After being transferred to 500 microliter aliquots, serum samples were stored at –20 degrees Celsius prior to permanent storage at –80 degrees Celsius until analysis.

Laboratory measurements

Laboratory measurements in serum samples were performed after all subjects had completed the study. Samples from 6 participants were measured as a pilot, followed by measurements in the remaining 23 participants' samples. All measurements were performed with the same lot number. For each participant, samples from the different time points were measured in the same batch.

Assays and assay performance

All measurements were performed with fully automated, software monitored equipment and diagnostics from Roche Diagnostics (Almere, The Netherlands) at the Department of Clinical Chemistry and Laboratory Medicine at Leiden University Medical Centre, The Netherlands. Aspartate aminotransferase (AST) (Catalogue number 11876848216), alanine aminotransferase (ALT) (Catalogue number 11876805216) and creatine (Catalogue number 5168589190) for estimating glomerular filtration rate (GFR) were measured from a fasted morning serum sample using the Modular P800 clinical chemistry analyser. GFR was calculated using the CKD-EPI

calculation. Thyroid parameters TSH (Catalogue number 11731459122, research reference identifier (RRID): AB_2756377), fT4 (Catalogue number 6437281190, RRID: AB_2801661), T4 (Catalogue number 12017709122, RRID: AB_2756378), fT3 (Catalogue number 6437206190, RRID: AB_2827368) and T3 (Catalogue number 11731360122, RRID: AB_2827369) were measured in serum by an immunoassay using Roche cobas8000 with an E602 module. The coefficients of variation (CV) were 2.36 (SD 0.52) for TSH, 5.55 (SD 2.28) for fT4, 2.06 (SD 0.58) for fT3, 5.25 (SD 0.34) for T3 and 2.88 (SD 0.41) for Tg.

Statistical Analyses

Descriptive statistics were used to summarise group characteristics. Independent samples T test, Mann-Whitney U test and Chi square test were used, depending on the characteristics of the variable (normally distributed, not normally distributed and categorical, respectively), to statistically test for differences between (male and female) F2-LLS and F2-Con regarding demographics, anthropometrics and laboratory measurements. We used log transformation to normally distribute data that were not normally distributed, or non-parametric testing in the case of data that could not be transformed to normal distribution. The cumulative area under the curve (AUC) was calculated using a trapezoid model with Matlab (Mathworks, Natick, MA). Here we used only the 72 h time point, thus the total integrated area. General linear modelling was used to investigate differences in TSH and TH kinetics between F2-LLS and F2-Con. In order to answer our research question on whether the thyroid gland of F2-LLS is less responsive to rhTSH stimulation than those of F2-Con, we calculated the ratio of total circulating thyroid hormones (AUC fT4, AUC fT3 and AUC Tg) to total circulating TSH during the study (AUC TSH). Pearson correlation was used to test the correlation between GFR and AUC TSH. Linear mixed modelling was used to test for differences in AUC ratios between F2-LLS and F2-Con adjusted for age and gender. In all analyses, $P \leq 0.05$ was considered statistically significant.

We used power calculations to determine group size. A two-sided significance level of 5% was used and the power was set at 80%. A sample size of 10 participants will have 80% power to measure a 2.71 pmol/L difference in fT4 levels after rhTSH stimulation. In addition, including 10 participants per group will have 92% power to detect a 0.73 pmol/L difference in fT3 levels after rhTSH stimulation. To overcome possible dropout due to difficulties with blood sampling or the laboratory measurements 15 participants were included in each group.

Programs used for statistical analyses were SPSS for Windows, version 23 (SPSS, Chicago, IL) and Matlab (The MathWorks Inc, Natick, MA). Graphs were made using Microsoft Office Excel 2016 and GraphPad Prism for Windows, version 8.1.1 (330) (GraphPad Software, Inc, San Diego, CA).

Results

Inclusions

The recruitment and inclusion flow chart of subjects for the study is presented in Figure 1. In total 83 individuals were selected and invited by telephone to participate. 13 individuals were not interested in receiving the informed consent form (ICF). Out of 70 individuals who did receive an ICF, 18 were not interested in participating in the study and 10 had significant medical history which made them unsuitable candidates. 42 individuals were included in the study and have undergone a medical screening. 12 individuals were excluded on the basis of the findings from the medical screening. Consequently, 30 subjects were included and have completed the study. The F2-LLS selected were non consanguineous. One participant was excluded from analyses due to suspected intravenous rhTSH administration based on TSH peak and concentration profile during the study. In this subject, the TSH peak of 243 mU/L ($>4SD$ from mean of other F2-LLS) was reached 15 min following 0.1mg rhTSH administration (versus on average 7 h in other F2-LLS). The TSH concentration subsequently remained $>4SD$ from the mean of other F2-LLS throughout study day

1, although subsequently decreasing and eventually reaching levels below baseline by study day 4.

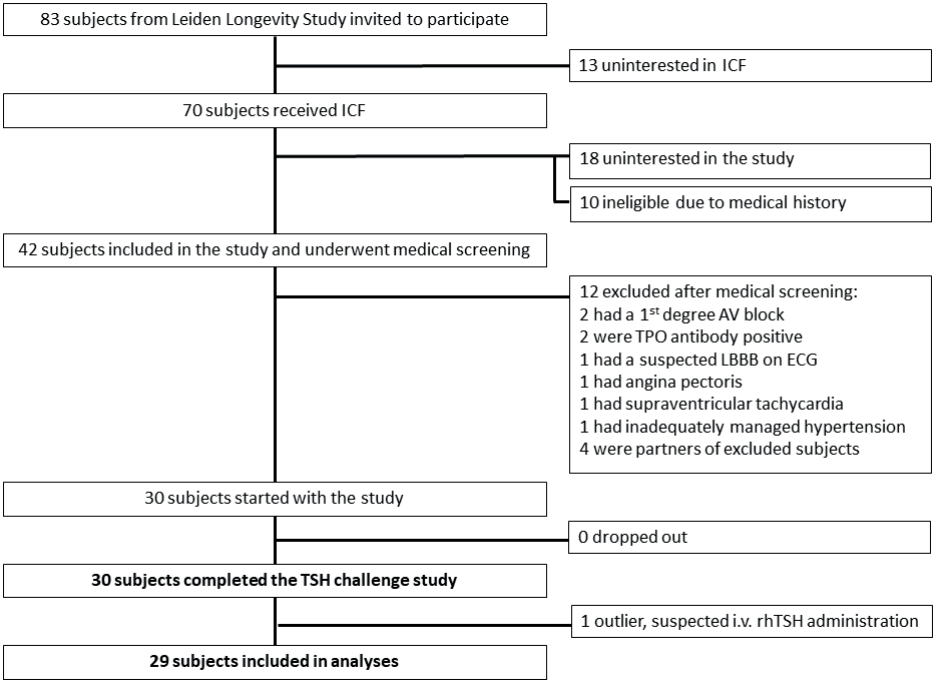


Figure 1. Recruitment and inclusion flowchart of subjects for the TSH challenge study. ICF: informed consent form, AV: atrioventricular, TPO: thyroid peroxidase, LBBB : left bundle branch block, ECG: electrocardiogram, i.v.: intravenous.

Group characteristics

Baseline characteristics of the study population are presented in Table 1. The F2-LLS (n=14) and F2-Con (n=15) were similar regarding age, sex and BMI. They comprised a healthy, high middle-age population. Parental age was higher in F2-LLS than in F2-Con (p<0.01 for mothers, and p=0.02 for fathers), confirming the longevity phenotype on which F2-LLS were selected for the Leiden Longevity Study. Both groups had a mean kidney function within normal range (GFR > 60 mL/min per

1.73m²), although kidney function was slightly lower in F2-LLS than in F2-Con ($p=0.04$). One participant, a female F2-LLS, had GFR 57 at screening, but was included in the study due to absence of any indication of chronic (kidney) disease. GFR was not significantly correlated with AUC TSH (Pearson correlation $r=-0.28$, $p=0.15$). Baseline TSH was significantly higher in F2-LLS than in F2-Con ($p=0.04$), while other thyroid hormones were similar between F2-LLS and F2-Con (fT4 $p=0.12$, fT3 $p=0.15$, Tg $p=0.18$), confirming the selection of participants with the longevity-associated TSH phenotype.

Table 1. Baseline characteristics of the study population.

	F2-LLS (n=14)	F2-Con (n=15)
DEMOGRAPHICS		
Age mother <i>years</i> *	93 (91–97)	75 (69–85)
Age father <i>years</i> *	93 (73–96)	78 (61–82)
Male <i>n (%)</i>	8 (57)	6 (40)
Age <i>years</i>	69 (5)	69 (6)
ANTHROPOMETRICS		
BMI <i>kg/m²</i>	25.8 (4.3)	26.3 (4.4)
Weight <i>kg</i>	78.1 (15.4)	78.1 (15.0)
Height <i>cm</i>	173.7 (10.9)	171.9 (9.0)
Fat mass <i>kg</i>	23.9 (7.2)	26.7 (8.4)
Lean mass <i>kg</i>	54.1 (12.9)	50.7 (13.2)
LABORATORY		
MEASUREMENTS	71.2 (13.9)	80.3 (8.4)
GFR <i>ml/min per 1.73m²</i>	22.3 (4.1)	24.6 (7.1)
AST <i>U/L</i>	19.6 (5.3)	22.1 (8.3)
ALT <i>U/L</i>	3.3 (1.7)	2.2 (1.0)
Baseline TSH <i>mU/L</i>	13.9 (13.0–15.8)	15.3 (14.3–15.7)
Baseline fT4* <i>pmol/L</i>	4.6 (0.5)	4.3 (0.5)
Baseline fT3 <i>pmol/L</i>	10.7 (6.9–22.9)	14.3 (10.2–33.6)
Baseline Tg* <i>µg/L</i>		

All values are mean (standard deviation) unless otherwise stated. F2-LLS: members of long-living families, F2-Con: partners of F2-LLS, BMI: body mass index, GFR: glomerular filtration rate, AST: aspartate transaminase, ALT: alanine transaminase, TSH: thyroid stimulating hormone, fT4: free T4, fT3: free T3, Tg: thyroglobulin.

*Median (interquartile range).

Thyroid response to rhTSH challenge

Following injection with 0.1mg rhTSH, circulating TSH levels increased to supraphysiologic levels in F2-LLS and F2-Con throughout study days 1, 2 and 3, and returned to baseline by day 4 (mean (SEM) in F2-LLS 2.6 (0.3) mU/L and in F2-Con 2.7 (0.3) mU/L), as shown in Figure 2A. The mean (SEM) peak TSH value was higher in F2-LLS than in F2-Con (34.5 (4.1) mU/L and 24.5 (2.7) mU/L, respectively; $p=0.047$), as shown in Figure 3A. Both peak values were reached on average 7 h after injection ($p=0.87$ between F2-LLS and F2-Con). Generalized linear model calculations show that circulating TSH was different between F2-LLS and F2-Con during the first 8 h following rhTSH administration, $p=0.031$, as well as different in time progression, $p<0.0001$. However, area under the curve calculations show that mean (SEM) AUC TSH for the whole study (72 h) was not significantly different between F2-LLS and F2-Con (985 (76) mU/L and 824 (57) mU/L, respectively, $p=0.10$).

Following 0.1mg rhTSH injection, thyroid hormones increased in both F2-LLS and F2-Con (Figure 2B-D) with most participants reaching peak values of fT4 and fT3 24 h after injection (interquartile range 24-48 and 8-24 h, respectively) and peak values of Tg 48 h after injection (interquartile range 48-48 h). Peak values of TH were similar in F2-LLS and in F2-Con (Figure 3B-D). Median (IQR) peak fT4 was 20.8 (19.4-24.5) pmol/L in F2-LLS and 24.2 (21.4-26.9) pmol/L in F2-Con, $p=0.07$. Mean (SEM) peak fT3 was 8.4 (0.5) pmol/L in F2-LLS and 9.1 (0.6) pmol/L in F2-Con, $p=0.36$. Median (IQR) peak Tg was 40.3 (22.7-64.0) $\mu\text{g/L}$ in F2-LLS and 50.5 (30.2-110.7) $\mu\text{g/L}$ in F2-Con, $p=0.16$. The whole study median (IQR) AUC for fT4 was 1411 (1276-1630) pmol/mL in F2-LLS and 1619 (1404-1713) pmol/mL in F2-Con, $p=0.10$. The whole study AUC fT3 was not significantly different between F2-LLS and F2-Con (mean (SEM) 506 (29) pmol/mL and 548 (34) pmol/mL in F2-Con, respectively, $p=0.36$). The whole study AUC Tg was not significantly different between F2-LLS and F2-Con (median (IQR) 2277 (1302-3126) $\mu\text{g/L}$ and 2977 (1804-6512) $\mu\text{g/L}$, respectively, $p=0.18$).

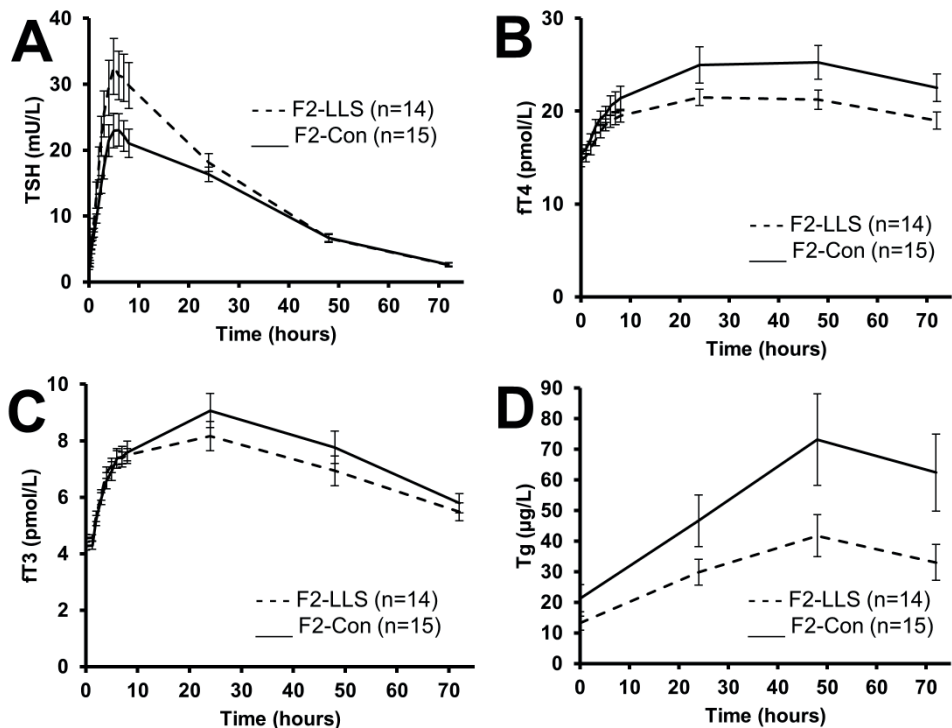


Figure 2. Four-day (72 h) profile of mean circulating thyroid stimulating hormone (TSH) and mean circulating thyroid hormones in members of long-lived families, F2-LLS (n=14) and their partners, F2-Con (n=15) following injection with 0.1mg recombinant human TSH. A) Mean circulating TSH, general linear model during first study day: p value = 0.031 between offspring and partners, within time p < 0.0001, offspring or partner over time p = 0.029, B) mean circulating free T4 (fT4), C) mean circulating free T3 (fT3) and D) mean circulating thyroglobulin (Tg). Black lines: F2-Con, dashed lines: F2-LLS. Error bars: standard error of the mean.

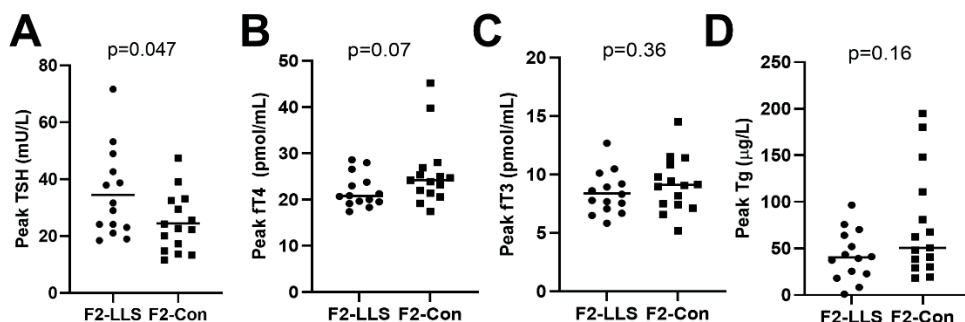


Figure 3. Peak values of circulating thyroid stimulating hormone (TSH) and circulating thyroid hormones in members of long lived-families, F2-LLS (n=14) and their partners, F2-Con (n=15) following injection with 0.1mg rhTSH. A) Peak values of TSH, horizontal line represents the mean, B) peak values of free T4 (fT4), horizontal line represents the median, C) peak values of free T3 (fT3), horizontal line represents the mean, and D) peak values of thyroglobulin (Tg), horizontal line represents the median. P value < 0.05 was considered statistically significant.

In order to investigate the response of the thyroid gland to TSH, we calculated the AUC fT4/AUC TSH ratio. The AUC fT4/AUC TSH ratio was lower in F2-LLS than in F2-Con at all time points, including baseline, as shown in Figure 4A (generalized linear model $p=0.04$ between F2-LLS and F2-Con). The whole study (72 h) AUC fT4/AUC TSH ratio was significantly lower in F2-LLS than in F2-Con (mean (SEM) 1.6 (0.1) pmol/mU and 2.2 (0.2) pmol/mU, respectively, $p=0.01$), as shown in Figure 4B. When adjusted for age and gender, the AUC fT4/AUC TSH ratio remained significantly different between F2-LLS and F2-Con (estimated mean (95% CI) 1.6 (1.2-1.9) pmol/mU and 2.2 (1.9-2.6) pmol/mU, respectively, $p=0.01$).

We investigated the relationship of secondary output parameters of the thyroid gland (fT3 and Tg) to TSH. We again observed the trend of a lower AUC fT3/AUC TSH ratio in F2-LLS than in F2-Con (Figure 5A), although the difference was not

statistically significant (mean (SEM) 0.6 (0.1) pmol/mU and 0.7 (0.1) pmol/mU, respectively, $p=0.07$). When adjusted for age and gender, the difference in AUC fT3/AUC TSH ratio between F2-LLS and F2-Con remained not significant (estimated mean (95% CI) 0.6 (0.4-0.7) pmol/mU and 0.7 (0.6-0.8) pmol/mU, respectively, $p=0.07$). The AUC Tg/AUC TSH (Figure 5B) was lower in F2-LLS than in F2-Con (median (IQR) 2.1 (1.4-3.6) $\mu\text{g}/\text{mU}$ and 3.2 (2.7-7.4) $\mu\text{g}/\text{mU}$, respectively, $p=0.04$). When adjusted for age and gender, the AUC Tg/AUC TSH ratio remained significantly different between F2-LLS and F2-Con (estimated mean (95% CI) 2.6 (1.1-4.0) $\mu\text{g}/\text{mU}$ and 4.8 (3.3-6.2) $\mu\text{g}/\text{mU}$, respectively, $p=0.04$).

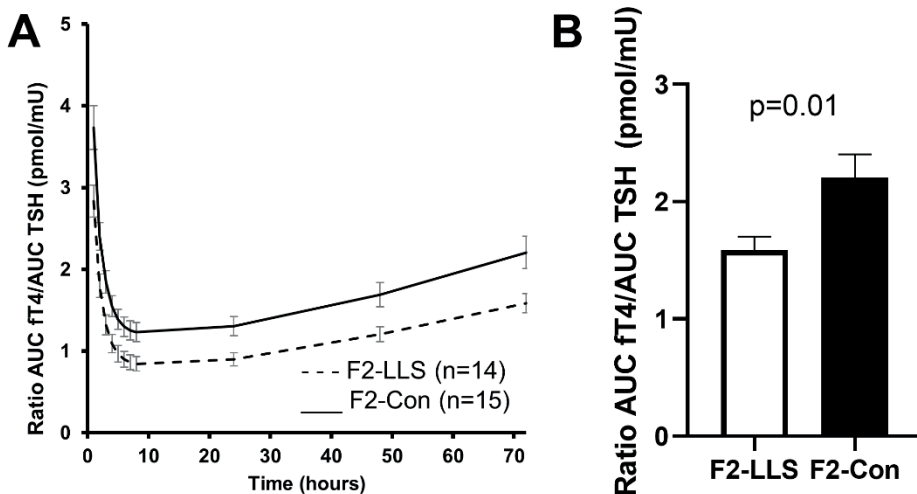


Figure 4. The ratio of circulating free T4 (fT4) to circulating thyroid stimulating hormone (TSH) in members of long-lived families, F2-LLS (n=14) and their partners, F2-Con (n=15), based on area under the curve calculations (AUC), following injection with 0.1mg recombinant human TSH. A) Four-day profile of the mean area under the curve ratio of circulating fT4 to circulating TSH. General linear modelling between offspring and partners p value = 0.04. B) Whole study (72 h) mean area under the curve ratio of circulating fT4 to circulating TSH. Error bars represent standard error of the mean.

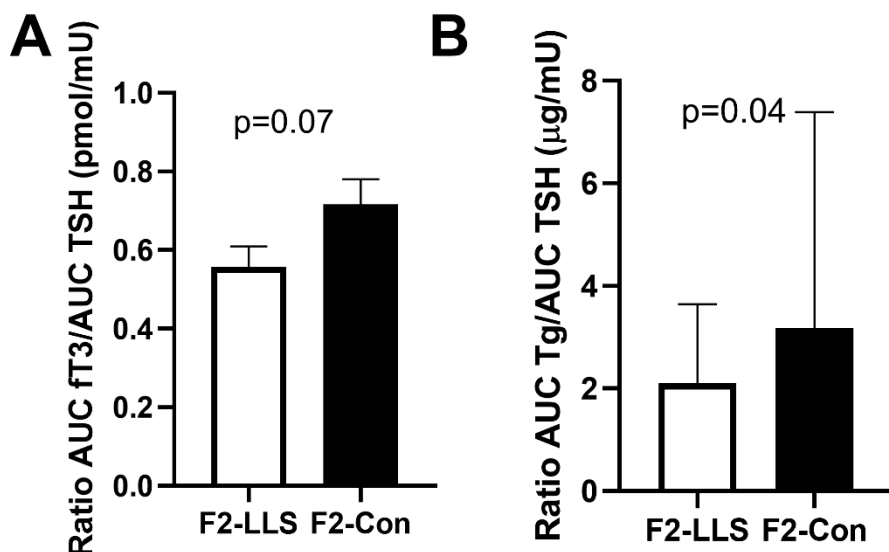


Figure 5. The ratio of circulating thyroid hormones free T3 (fT3) and thyroglobulin (Tg) to circulating thyroid stimulating hormone (TSH) in members of long-lived families, F2-LLS (n=14) and their partners, F2-Con (n=15), based on whole study (72 h) area under the curve calculations (AUC), following injection with 0.1mg recombinant human TSH. A) Mean area under the curve ratio of circulating fT3 to circulating TSH in offspring and partners, $p=0.07$, and B) median area under the curve ratio of circulating Tg to circulating TSH in offspring and partners, $p=0.04$. P value < 0.05 was considered statistically significant. Error bars represent standard error of the mean in A, and interquartile range in B.

Discussion

In this study, we investigated whether human familial longevity is associated with lower thyroidal responsivity to stimulation by TSH. Overall, we demonstrated that administration of 0.1mg rhTSH results in lower fT4 to TSH ratio in members from long-living families compared to their partners, thereby supporting our hypothesis that longevity is associated with lower thyroidal responsivity to TSH.

Whereas both F2-LLS and F2-Con had circulating fT4, fT3 and Tg levels within normal range throughout the study, F2-LLS had a lower AUC fT4/AUC TSH than F2-Con. This was the case at baseline, but also under challenge conditions, indicating the perseverance of lower thyroid responsivity in F2-LLS compared to F2-Con even in the presence of supraphysiologic circulating TSH levels. Secondary parameters of the thyroid gland, namely fT3 and Tg, showed a similar trend, with the AUC fT3/AUC TSH non significantly lower and AUC Tg/AUC TSH significantly lower in F2-LLS compared to F2-Con.

TSH and TH profiles upon administration of 0.1mg rhTSH in our study were comparable to those from previous studies concerning the use of rhTSH in healthy young and middle-aged subjects (10-14). The dose of 0.1mg rhTSH was adequate to increase circulating TSH to supraphysiologic levels in young and middle-aged subjects, where levels of circulating TSH increased two hours after intramuscular injection with rhTSH and return to baseline over the course of 3 to 4 days (10,11,14), corresponding to TSH concentration profiles in our study. Peak TSH values following injection were variable in healthy young and middle-aged adults (14-16), as was also the case in our study. Despite reached supraphysiologic levels of circulating TSH, there were no serious adverse events (SAEs) or suspected unexpected serious adverse reactions (SUSARs) in our study, indicating the safety of this dose in healthy older individuals, which could at least in part be due to sustained circulating TH within the normal range, as previously also reported (10,14). Upon administration of the same dose of rhTSH, TSH concentrations over the first 8 hours (including peak TSH concentration) were higher in F2-LLS than in F2-Con. We have not found an explanation for this result and its implications remain unclear. Importantly, it has previously been reported that TSH concentrations following injection with rhTSH vary widely between individuals, and that these differences may be influenced by gender, age and body composition(14,15). In our study, age, BMI and gender distribution were comparable between the groups of F2-LLS and F2-Con. In addition, we did not find a significant

difference in TSH peak concentrations after stimulation with rhTSH between men and women.

This study for the first time provides a mechanistic underpinning for the previously observed higher circulating TSH but similar TH levels in members of long-lived families compared to controls (5). Although the combination of higher TSH with similar levels of TH has not yet been studied in animal models of longevity, previous findings have reported negative associations between thyroid hormone levels and lifespan in multiple animal models (1,17). Interestingly, long-lived Ames and Snell mutant dwarf mice (18,19), which exhibit a combined hormonal deficiency for GH, TSH and prolactin show traits that are related to thyroid hormone deficiency, and supplementation of thyroid hormone during adulthood partly reduces their increased life span (20).

The study has several strengths. Firstly, the high frequency of blood sampling following administration of 0.1mg rhTSH allowed for observation in great detail of TSH pharmacokinetics during the first 8 h after administration. Secondly, careful planning of laboratory measurements allowed for minimal inter-measurement variation – all samples were measured once all participants have completed the study, and the potential confounding effect of laboratory batch variation was avoided by using the same reagent and the same batch for all samples per participant. Finally, to study the mechanism underlying altered thyroid phenotype in familial longevity, we have selected participants from the pool of F2-LLS from long-living families and their partners in whom this difference in TSH phenotype has previously been found (5), thereby investigating this specific mechanism in the specific target population, which is a major strength of the study. Additionally, our study had strict health criteria in order to minimise any risk of adverse events and side effects in our high middle-age population under challenge conditions. This means we have selected a relatively healthy group of older individuals, with minimal confounding by comorbidities or polypharmacy, allowing us to optimally study the physiological effect of rhTSH on these subjects. Although the sample size was small it was adequate to detect

differences in fT4 levels between F2-LLS and F2-Con following 0.1mg rhTSH administration, while remaining ethically responsible due to high burden of the study for the participants.

The study has a couple of limitations. Firstly, to minimise recruitment bottlenecks, we did not include a placebo control group to observe thyroid parameters throughout the study period without intervention. Hence, we cannot adjust for baseline thyroid parameters throughout the study. It is possible that the difference we have found between F2-LLS and F2-Con is therefore underestimated and would be even greater when adjusted for physiological thyroid values. Secondly, although conditions during study day 1 were standardized (fixed meal times, standardised meals, only water or tea for beverages), there were no alcohol abstinence guidelines during the remainder of the study. Since rhTSH is at least partly metabolised by the liver, it is possible that alcohol consumption during the remainder of the study has influenced TSH pharmacokinetics in some subjects. However, since most of the population consumed 1 to 2 glasses of alcohol per day in general and subjects consuming >4 glasses of alcohol per day were not included in the study, the effect of alcohol on TSH kinetics is probably minimal. In future clinical studies with drugs metabolised by the liver, we recommend advising alcohol abstinence during the study or documenting alcohol consumption.

The principal finding of this study is that familial longevity is associated with lower thyroid responsivity to TSH. In future studies, we aim to investigate the influence of this finding on secondary tissues influenced by the thyroid axis, by measuring parameters of bone turnover, and different immune parameters, in order to investigate possible extrathyroidal effects of TSH, since It has been speculated that TSH has direct effects on tissues other than the thyroid gland. This hypothesis is supported by the observation that the TSH receptor is expressed in cells from several other tissues than the thyroid gland, including bone, adipose tissue, brain and thymus (21-25). Moreover, other mechanisms underlying elevated TSH levels in the absence of elevated thyroid hormones in F2-LLS from long-living families are possible. TSH

bioactivity in these subjects has been tested and has been found to be similar between F2-LLS and F2-Con (5), but higher TH turnover in F2-LLS compared to F2-Con has not yet been investigated. Further studies, possibly through administration of TH in members of long-living families and their partners, are needed to investigate this.

In summary, familial longevity is associated with attenuated thyroid responsivity to an rhTSH challenge as observed by lower AUC fT₄/TSH and AUC Tg/TSH ratio in members from long-lived families compared to controls. Further studies investigating extrathyroidal effects of TSH as well as turnover of TH in familial longevity are necessary to advance our understanding of the role of the thyroid axis in longevity.

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Data Availability

The datasets generated during and analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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Chapter 5

Circulating Thyroid Hormone Profile in Response to a Triiodothyronine Challenge in Familial Longevity.

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Abstract

CONTEXT. Familial longevity is associated with higher circulating levels of thyroid stimulating hormone (TSH), in the absence of differences in circulating thyroid hormones, and a lower thyroid responsivity to TSH, as previously observed in the Leiden Longevity Study (LLS). Further mechanisms underlying these observations remain unknown.

OBJECTIVE. We hypothesized that members from long-lived families (offspring) have higher thyroid hormone turnover or less negative feedback effect on TSH secretion compared to controls.

METHODS. In a case-control intervention study, 14 offspring and 13 similarly aged controls received 100 µg triiodothyronine (T3) orally. Their circulating T3, free T3 (fT3) and TSH levels were measured during five consecutive days. We compared profiles of circulating T3, fT3 and TSH between offspring and controls using general linear modelling (GLM) and calculated the percentage decline in TSH following T3 administration.

RESULTS. Circulating T3 and fT3 levels increased to supraphysiologic values, and normalized over the course of 5 days. There were no serious adverse events. T3 and fT3 concentration profiles over 5 days were similar between offspring and controls (T3 GLM $p=0.11$, fT3 GLM $p=0.46$). TSH levels decreased in a biphasic manner and started returning to baseline by day 5. The TSH concentration profile over 5 days was similar between offspring and controls (GLM $p=0.08$), as was the relative TSH decline (%).

CONCLUSIONS. Members of long-lived families have neither higher T3 turnover nor diminished negative feedback of T3 on TSH secretion. The cause and biological role of elevated TSH levels in familial longevity remain to be elucidated.

Precis

The results from a challenge study with 100 µg triiodothyronine in 27 euthyroid participants indicate that T3 turnover as well as negative feedback of T3 on TSH are not different between members of long-lived families and similarly aged controls.

Introduction

Thyroid stimulating hormone (TSH) levels tend to increase with age (1). It is unknown to what extent this increase reflects selective survival of people with a genetic predisposition for relatively higher TSH (2), and to what extent it reflects an adaptive process.

In the Leiden Longevity Study (LLS) it has previously been observed that members of long-lived families (offspring) had on average 0.8 mU/L higher TSH levels than similarly-aged controls throughout a 24-h period, whereas thyroid hormones were not different between the groups (3). This discrepancy could not be explained by a reduced bioactivity of TSH, which has been shown to not differ between offspring and controls (3).

This study is one of the two studies we performed in parallel to further understand the mechanisms behind higher TSH in offspring than controls in the presence of similar thyroid hormone levels. We have recently shown that offspring have lower thyroidal response to TSH by performing a challenge study with recombinant human TSH (4). We hypothesized that offspring might also have higher turnover of thyroid hormones and/or less negative feedback on TSH.

To investigate this, we performed a single challenge study with 100 µg triiodothyronine (T3) in offspring from long-lived families and controls. We were not expecting complete inactivating receptor defects of the thyroid hormone receptor or activating mutations of deiodination enzymes or enzymes of the sulphating pathways, but rather subtle defects as found in polymorphisms. We hypothesized that upon administration of a single dose of T3, offspring would have a higher decrease in

concentration profiles of circulating T3 and fT3 than controls and less negative feedback of T3 on TSH.

We believe that these mechanisms are not mutually exclusive but could in concert be contributing to the previously observed differences in thyroid axis phenotype between offspring and controls.

Materials and methods

Study population

The Leiden Longevity Study was founded in 2002 and designed to investigate genotypes and phenotypes underlying inter-individual differences in familial longevity in humans (5). Subjects recruited into the study were siblings from 421 Caucasian long-living families (men aged 89 and older, women aged 91 and older) living in The Netherlands in the early 2000's, without any restrictions on health or demographics (6). The offspring of these families, who were also found to have a lower morbidity than controls (6), were also asked to participate in the study, with their current partners as reference group, thereby creating a case group enriched for longevity (offspring) and a reference group with similar lifestyle factors and socio-economic status as the case group, but without selection for familial predisposition to longevity (controls).

Subjects were recruited for the T3 challenge study from the subgroup of LLS previously studied in terms of thyroidal status between offspring and controls (3), and excluded based on: laboratory results (haemoglobin < 7.1 mmol/L, TSH > 4.0 mU/L, fT4 < 9 pmol/L or > 24 pmol/L, TPO antibody positivity (>35 kU/L)), medical history (cardiac arrhythmias, (history of) thyroid diseases, renal, hepatic or endocrine disease, or any other significant chronic disease), medication use (hormone therapy, thyroid medication), lifestyle factors (nicotine abuse, (history of) alcohol abuse (>28 units per week)) and practical factors (difficulty inserting an intravenous cannula, participation in other research projects within the last 3 months, participation

in 2 or more projects in one year, evaluation by physician as too vulnerable to participate). Our subjects had no palpable goitre.

Clinical protocol

Participants were admitted into the study after passing the medical screening. The study consisted of five consecutive study days at Leiden University Medical Centre. On the morning of study day 1, subjects were in the fasted state, an intravenous cannula was placed in a forearm vein, blood was withdrawn at baseline and 100 µg (4 x 25 µg) triiodothyronine was administered orally with a glass of water. The time of administration was used as reference, time zero. Thereafter, blood was sampled at high frequency: every 15 min in the first four hours after administration, every 30 min between 4 and 7 h after T3 administration, and finally every hour between 7 and 9 h after T3 administration. During this time, subjects received three standardized meals (after the 2-h blood sample, 5-h blood sample and 8-h blood sample), each consisting of 600 kcal (2x125mL Nutridrink Compact, Nutricia Advanced Medical Nutrition, Zoetermeer, The Netherlands). On study day 2, 3, 4 and 5, additional fasted blood samples were obtained at respectively 24, 48, 72 and 96 h after T3 administration. Subjects were at their leisure outside of these times.

In total, 370 ml of blood was withdrawn from each subject across 29 time points (25 on study day 1, and one on days 2, 3, 4 and 5).

Height, weight and body composition were measured on study day 2. Body composition was measured with a Bioelectrical Impedance Analysis meter at a fixed frequency of 50kHz (Bodystat 1500 Ltd, Isle of Man, British Isles(7)).

The study was designed in accordance with the declaration of Helsinki and has been approved by the Medical Ethical Committee of the Leiden University Medical Centre. It is registered at Leiden University Medical Centre under the protocol P16.107 and with EudraCT under the number 2016-001497-15. All subjects gave written informed consent prior to the screening visit.

Handling of samples

Serum samples were kept at room temperature for 60 min to clot before being processed at the Clinical Chemistry and Laboratory Medicine Department, Leiden University Medical Centre, The Netherlands. Samples were centrifuged for 10 min at 2350 G relative centrifugal force at a temperature of 20 degrees Celsius. After being transferred to 500 microliter aliquots, serum samples were temporarily stored at –20 degrees Celsius prior to permanent storage at –80 degrees Celsius until analysis.

Laboratory measurements

Laboratory measurements were performed after all subjects had completed the study. We first measured samples from 6 participants as a pilot to identify whether T3 and fT3 were increased and at which time points, followed by measurements of samples from the remaining 21 participants at all time points. All measurements were performed with the same lot number. All samples from one individual were measured in the same batch.

Assays and assay performance

Laboratory measurements were performed with fully automated, software monitored equipment and diagnostics from Roche Diagnostics (Almere, The Netherlands) at the Clinical Chemistry and Laboratory Medicine Department at Leiden University Medical Centre, The Netherlands. Aspartate aminotransferase (AST) (Catalogue number 11876848216), alanine aminotransferase (ALT) (Catalogue number 11876805216) and creatine (Catalogue number 5168589190) were measured from a fasted morning serum sample using the Modular P800 clinical chemistry analyser. Creatine values were used to estimate glomerular filtration rate (GFR) using the CKD-EPI calculation. Thyroid parameters TSH (Catalogue number 11731459122, research reference identifier (RRID): AB_2756377), fT4 (Catalogue number 6437281190, RRID: AB_2801661), T4 (Catalogue number 12017709122, RRID: AB_2756378), fT3 (Catalogue number 6437206190, RRID: AB_2827368) and T3 (Catalogue number

11731360122, RRID: AB_2827369) were measured in serum by an immunoassay using Roche cobas8000 with an E602 module. The coefficient of variation (CV) for TSH was 2.36% (SD 0.52), for fT4 5.55% (SD 2.28), for fT3 2.06% (SD 0.58), for T3 5.25% (SD 0.34) and for Tg 2.88% (SD 0.41).

Statistical Analyses

Descriptive statistics were used to summarise group characteristics. Independent samples T test, Mann-Whitney U test and Chi square test were used, depending on the characteristics of the variable (normally distributed, not normally distributed and categorical, respectively), to statistically test for differences between offspring and controls regarding demographics, anthropometrics and laboratory measurements. General linear modelling (GLM) was used to investigate differences in the concentration profiles of T3, fT3 and TSH between offspring and controls. In all analyses, $P \leq 0.05$ was considered statistically significant.

Previous studies have investigated the effect of T3 administration on thyroid function in healthy individuals Spencer et al. showed that oral administration of a single dose of 100 µg synthetic T3 significantly suppressed TSH levels (8). It was hypothesized that controls would show the same pattern in T3 and TSH levels after T3 administration as was observed in the study performed by Spencer et al. while for the offspring a faster decrease in T3 levels was expected. Consequently, for TSH levels it was hypothesized that offspring will show higher levels compared to controls due to the faster disappearance of T3. Based on the abovementioned assumptions a sample size calculation was performed to compare the expected area under the curve (AUC) in the TSH suppression curves of offspring and controls. For the sample size calculation, a SD of 30.6 was used, which is two times the SD of the AUC 24-hours TSH for the controls as observed in Switchbox (3).

A two sided significance level of 5% was used and the power was set at 80%. Based on these assumptions a sample size of 10 participants in each group was needed to measure an 1.93 times larger AUC TSH in offspring compared to controls after T3

administration with 80% statistical power. To overcome possible dropout due to difficulties with blood sampling or the laboratory measurements, we aimed to include 15 participants in each group.

Programs used for statistical analyses were SPSS for Windows, version 23 (SPSS, Chicago, IL), Systat version 13 (Systat Software, Inc, San Jose, CA) and Matlab (The MathWorks Inc, Natick, MA). Graphs were made using Microsoft Office Excel 2016 and GraphPad Prism for Windows, version 8.1.1 (330) (GraphPad Software, Inc, San Diego, CA).

Results

Inclusions

The recruitment and inclusion flow chart is presented in Figure 1. Thirty-eight individuals were invited by telephone to participate. Two of these individuals were not interested in receiving the informed consent form (ICF). Out of the 36 individuals who did receive an ICF, 6 were not interested in participating in the study. Thirty individuals were included in the study and have undergone a medical screening. Three individuals were excluded based on the screening findings (1 had a solely financial motivation to part-take, making it unadvisable to include this person under Dutch Good Research Practice, 1 was diagnosed with a first degree atrioventricular block, 1 had TPO antibody positivity) and twenty-seven subjects were included and went on to complete the T3 challenge study.

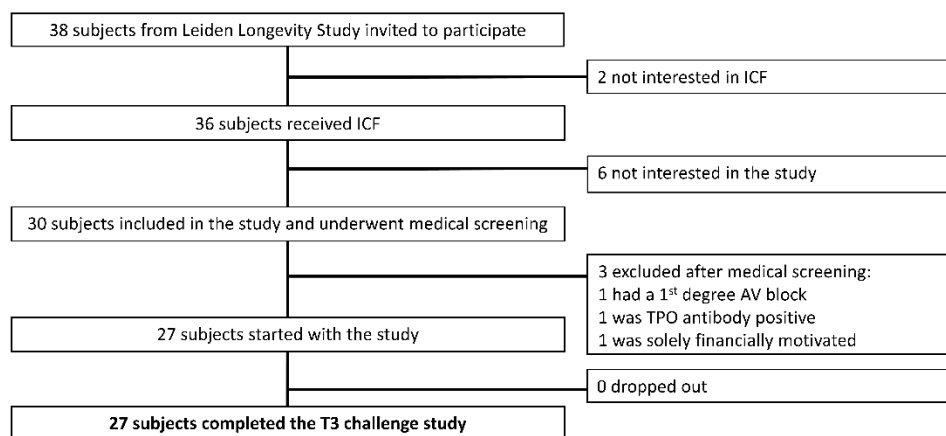


Figure 1. Recruitment and inclusion of subjects for the T3 challenge study. ICF: informed consent form, AV: atrioventricular, TPO: thyroid peroxidase.

Group characteristics

Baseline characteristics of the study population are presented in Table 1. Our study population consisted of healthy older middle-aged participants. The offspring ($n=14$) and control ($n=13$) groups were similar in terms of age, sex and body mass index (BMI). Maternal age was higher in offspring than in controls ($p < 0.01$), confirming the longevity phenotype in respect of which offspring was selected for the LLS. The same trend was observed in paternal age, although this difference was not statistically significant ($p=0.06$). Liver and kidney function were within normal range in all subjects and were similar in offspring and controls. All participants were clinically euthyroid at baseline, with higher TSH and similar fT4 levels in offspring and controls (TSH $p=0.05$, fT4 $p=0.17$). The fT3/fT4 ratio at baseline was higher in offspring than in controls ($p=0.005$).

Table 1. Baseline characteristics of the T3 challenge study.

	Offspring (n=14)	Controls (n=13)	P-value
Age mother (yr)*	94 (91-97)	75 (70-87)	0.003
Age father (yr)*	94 (73-96)	78 (68-84)	0.06
Men n (%)	9 (64)	5 (36)	0.18
Age (yr)	69 (67-72)	70 (66-72)	0.63
BMI (kg/m ²)	25.5 (3.8)	26.2 (4.3)	0.61
Weight (kg)	79.0 (14.3)	78.3 (14.1)	0.96
Height (cm)	175.6 (10.2)	172.5 (9.0)	0.54
Fat mass (kg)	22.7 (6.3)	26.0 (9.8)	0.31
Lean mass (kg)*	59.7 (42.1-64.8)	52.3 (43.9-65.1)	0.58
GFR (ml/min/1.73 m ²)	75.2 (13.5)	77.8 (8.2)	0.56
AST (U/L) *	21.7 (19.8-24.0)	23.0 (20.0-26.8)	0.34
ALT (U/L)	19.9 (5.8)	20.5 (5.0)	0.80
TSH (mU/L)	2.6 (1.2)	2.0 (1.1)	0.05
fT4 (pmol/L)	14.3 (2.1)	15.1 (1.9)	0.26
fT3 (pmol/L)	4.39 (0.36)	4.02 (0.49)	0.03
fT3/fT4 ratio	0.313 (0.046)	0.267 (0.029)	0.005

Data are shown as mean (standard deviation) or as median (interquartile range).

Abbreviations: BMI: body mass index, GFR: glomerular filtration rate, AST: aspartate transaminase, ALT: alanine transaminase, TSH: thyroid stimulating hormone, fT4: free T4. *Median (interquartile range). ** In offspring, based on 13 values due to one missing sample.

Circulating T3 and fT3 following T3 administration

Following oral administration of 100 mcg T3, circulating T3 and fT3 levels increased to supra physiologic levels in both offspring and controls, reaching peak concentrations during study day 1 and subsequently decreasing in concentration and returning to baseline levels by study day 5 (Figure 2A-B). The maximum concentration of T3 was not different between offspring and controls (mean (SEM) 12.5 (0.2) and 12.8 (0.2) nmol/L, respectively, $p=0.22$) and the same was true for fT3 (mean (SEM) 37.6 (0.2) and 37.3 (0.4) pmol/L, respectively, $p=0.84$). In both groups, the maximum concentration of T3 was reached 2 hours (median) following T3 administration (interquartile range (IQR) 1.75-2.00, $p=0.75$ between offspring and controls), and the maximum concentration of fT3 was reached 2.25 hours (median) following T3 administration (IQR 2 – 2.30, $p=0.84$ between offspring and controls).

Circulating T3 and fT3 levels were similar between offspring and controls throughout the study (GLM $p = 0.11$ for T3, GLM $p = 0.46$ for fT3) and variable over time in both groups (GLM $p<0.0001$). There was no difference in the concentration profiles of T3 and fT3 over time between the two groups (GLM $p=0.44$ for T3 and $p=0.52$ for fT3). In addition, serum T3 concentrations after reaching its maximal concentration were fitted to a bi-exponential function, $y=a e^{-ct} + b e^{-dt}$. No differences between the kinetic parameters in the offspring and control group were present (Table 2).

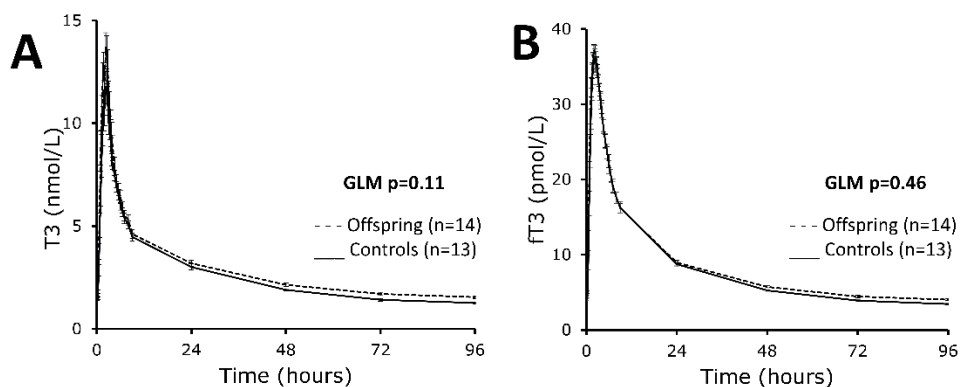


Figure 2. Five-day (96 h) profile of mean circulating triiodothyronine (T3) and free T3 (fT3) in members of long-lived families, offspring (n=14) and similarly aged controls, controls (n=13) following a challenge with 100 µg triiodothyronine (T3) (oral administration). A) Whole study (96 h) mean circulating T3 profile as measured across 29 time points, general linear modelling (GLM): p value=0.11 between offspring and controls, $p<0.0001$ in time, $p=0.44$ for time x group. B) Whole study (96 h) mean circulating fT3 profile as measured across 29 time points, GLM: p value = 0.46 between offspring and controls, $p<0.0001$ in time, $p=0.52$ for time x group. Dashed lines: offspring, solid lines: controls. Error bars: standard error of the mean.

Circulating TSH following T3 administration

Following T3 administration, TSH levels decreased in a biphasic manner with an acute decrease phase during study day 1 and a more gradual decrease on study days 2, 3 and 4, with levels returning to baseline by day 5 (Figure 3). The nadir levels of TSH were similar between offspring and controls (mean (SEM) 0.281 (0.008) and 0.215 (0.012) mU/L, respectively, $p=0.19$) and were reached around 48 hours (median) after T3 administration in both groups (IQR 48-48 h in offspring and 48-72 h in controls, $p=0.48$). The 96-h concentration profile of circulating TSH was similar between offspring and controls throughout the study (GLM $p=0.08$ between offspring and

controls). Baseline TSH levels between offspring and controls differed less than in the previous study (0.6 versus 0.8 mU/L) resulting in a borderline statistical difference (p=0.05). We had previously observed that offspring have higher circulating TSH levels than controls as a part of their familial longevity phenotype. Therefore, we calculated the percentage decline in TSH throughout the study relative to baseline TSH to further account for any potential difference in TSH levels between offspring and controls. The relative TSH decline (%) was similar between offspring and controls throughout the study period (Figure 4).

Table 2. Kinetic parameters of exponential fitting the declining serum concentrations of T3 after administration of 100 µg T3.

	Offspring (n=14)	Controls (n=13)	P-value
Coefficient a (nmol/L)	8.82±0.79	7.76±0.45	0.26
Coefficient b (nmol/L)	4.60±0.26	4.49±0.33	0.78
Slow rate constant (h ⁻¹)	0.015±0.001	0.016±0.001	0.47
Fast rate constant (h ⁻¹)	0.984±0.003	0.980±0.003	0.39

Data are shown as mean ± SEM. The formula used for the fitting was $y=a*e^{-c*t}+b*e^{-d*t}$

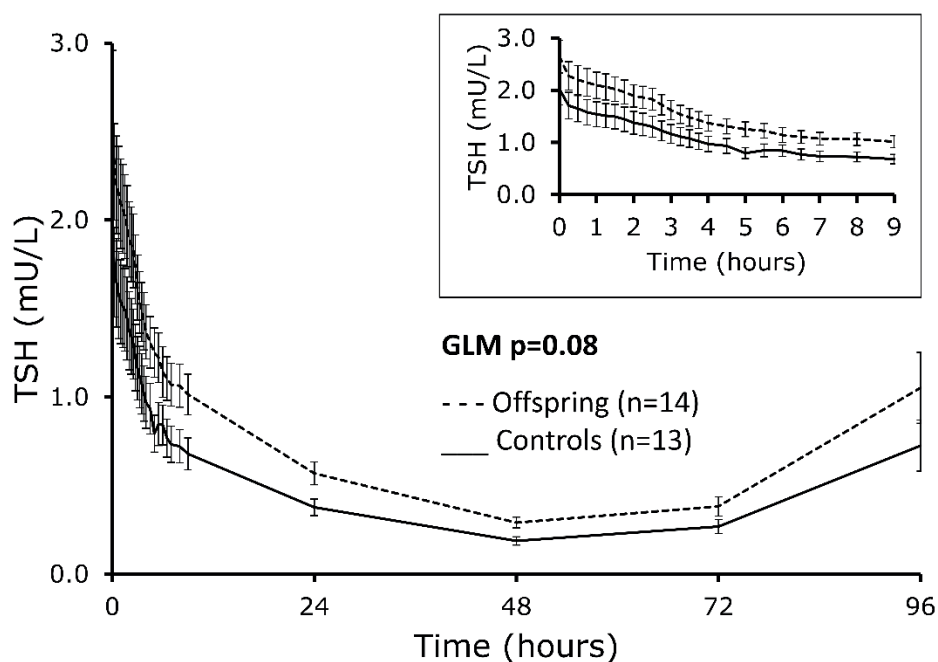


Figure 3. Five-day (96 h) mean circulating thyroid stimulating hormone (TSH) following a challenge with 100 µg triiodothyronine (T3, oral administration) in members of long-lived families, offspring (n=14) and similarly-aged controls (n=13), as measured across 29 time points. General linear modelling: p value=0.08 between offspring and controls. Inlay: detail of mean TSH profile during study day 1. Dashed lines: offspring, solid lines: controls. Error bars represent the standard error of the mean.

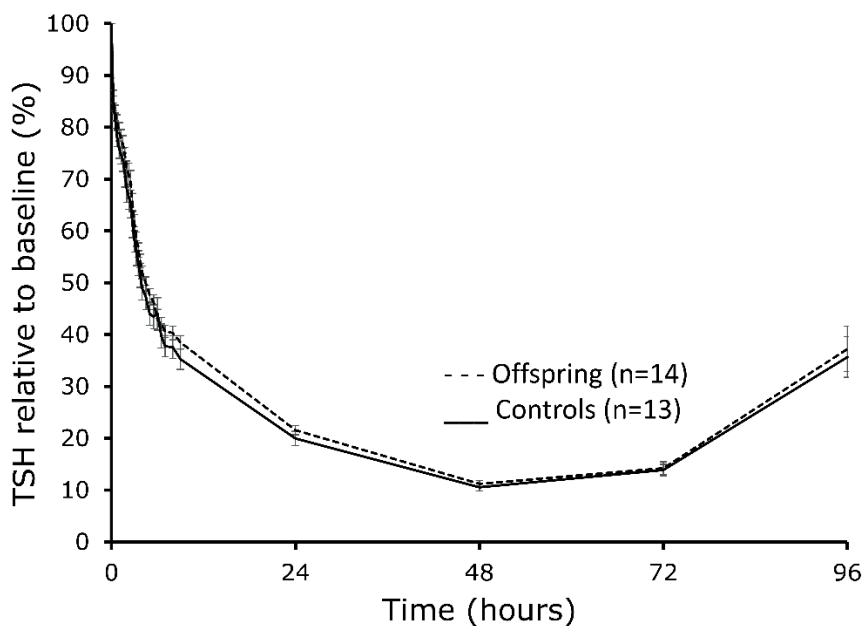


Figure 4. Five-day (96 h) profile of circulating thyroid stimulating hormone (TSH), expressed as the percentage relative to baseline, following a challenge with 100 mcg triiodothyronine (T3, oral administration) in members of long-lived families, offspring (n=14) and similarly aged controls (n=13), as measured across 29 time points. Dashed lines: offspring, solid lines: controls. Error bars represent the standard error of the mean.

Discussion

In this study, we investigated two hypotheses related to the thyroid axis in familial longevity: whether familial longevity is associated with higher T3 turnover and/or lower negative T3 feedback on TSH. Following a challenge with 100 µg T3 orally, we found that T3 and fT3 concentration profiles, as well as relative TSH decline, were similar between members of long-lived families and controls.

This is the first time that a single challenge study with such a potent dose of T3 has been performed in an older middle-aged population. Circulating T3 and fT3 increased to supra-physiological levels in proportion to those reported in healthy young adults with other doses (8-10). As expected, the 100 µg T3 dose did not cause any serious adverse events (SAEs), and no suspected unexpected serious adverse reactions (SUSARs) were reported in our study, despite the transient high circulating T3 and fT3 levels, indicating the safety of this dose in a single administration in healthy, euthyroid, older middle-aged individuals.

We based the dose of oral T3 on a previously published dose-response curve study, which used doses ranging from 40 to 1000 µg T3 (8), in conjunction with power calculations regarding the required sample size for several doses. We chose the dose of 100 µg due to multiple reasons. First of all, treatment with synthetic T3 might be associated with undesirable effects such as dizziness, headache, hot flushes, sweating, weight gain, and heart palpitations with irregular heartbeat. These side effects were not reported using a single dose of 100 µg T3 (8). Additionally, using a 100 µg T3 dose, our power calculations indicated a sample size sufficient to assess the underlying hypothesis of the study which was feasible within the given timeline and study design. Using lower doses would have required a higher sample size, which might have caused recruitment bottle necks.

Throughout the study, we observed comparable concentration profiles of circulating T3 and fT3 in offspring and controls, indicating that T3 turnover is likely similar in these two groups, at least under challenge conditions. Additionally, the levels of T3 and fT3 recovered to baseline in both groups, corresponding to the trend previously observed in young adults (9,10). In fact, T3 was chosen as an intervention for this study on the basis of its relatively short half-life compared to the other thyroid hormone, thyroxine (T4). Previous studies in healthy subjects found that T3 levels increase and TSH levels decrease within a few hours following oral T3 administration (8,10), and that the half-life of T3 is about 6 hours to 1 day (9,11). In contrast, T4 has

a half-life of about 4 to 6 days (9,11), making it a less suitable candidate for a short-term clinical study.

We calculated the percentage decline of TSH relative to baseline in order to investigate the negative feedback on TSH following T3 administration in offspring and controls. Circulating TSH declined in a similar way in offspring and controls, consistent with the (biphasic) decline previously reported in the literature (9,10). Our findings also confirm the previously reported TSH –T3 relationship observed in members from long-lived families compared to controls: in physiological conditions members from long-lived families have a stronger temporal relationship between TSH and free T3 than controls, in the absence of differences in the negative feedback by thyroid hormones on TSH (12). We confirmed this here by showing a comparable decline in TSH levels in the presence of similar circulating T3 and fT3 levels. However, under physiological conditions T4 rather than T3 is the feedback signal for the HPT-axis, acting on the pituitary thyrotrope and the hypothalamic tanycyte, involved in the secretion of thyrotropin-releasing hormone (TRH), by local conversion of T4 in T3 by deiodinase D2 (13). The demonstration that exogenous T3, leading to relatively high serum T3 concentrations, inhibits TSH secretion to a similar extent in offspring and controls, does not necessarily exclude dissimilarities in feedback by low-dose exogenous T4, and further experiments are indicated to confirm or refute this possibility.

Altogether, several findings provide clues regarding the mechanisms that might underlie the differences in thyroid axis (higher TSH levels in the absence of differences in thyroid hormones) between members of long-lived families and controls. We recently performed a challenge study with recombinant human TSH in a subpopulation of the LLS, showing that members of long-lived families have a lower thyroidal responsivity to TSH stimulation than similarly aged controls (4). In this study, the findings point to similar T3 turnover and similar negative T3 feedback control of TSH secretion in offspring and controls. In concert, these findings indicate that higher circulating TSH in offspring is not a result of less negative feedback by T3

on the pituitary, or a peripheral effect caused by higher turnover of T3. Rather, the primary mechanism behind the thyroid phenotype previously observed in offspring seems to be in the thyroid gland, in which lower responsivity to TSH requires higher circulating TSH levels to produce adequate quantities of thyroid hormones, a condition resembling early autoimmune thyroid failure, leading to subclinical hypothyroidism, characterized by normal serum T4 and high normal serum TSH concentrations.

The fT3/fT4 ratio was significantly higher in offspring, where this change could be caused by decreased T3 clearance or increased deiodinase D2 activity in organs such as the liver, kidney and muscle. The declining serum T3 levels following T3 maximum, as well as the kinetic parameters fitted by a biexponential model, were comparable in offspring and controls. Therefore, the increased ratio likely reflects increase D2 deiodinase activity. This finding was previously reported in 805 nonagenarians from the Leiden longevity study and 259 nonagenarians of the Leiden 85+ study, where higher fT3/fT4 ratio (and higher fT3 and lower fT4) were associated with lower mortality rate, independent of familial longevity status (14). Apparently, a higher ratio implies a healthier status, but at this time any mechanistic explanation is speculative. Our findings agree with a recent Italian study, where centenarians with a lower fT3/fT4 and higher fT4 levels have an impaired functional status and increased mortality (15).

Interestingly, an alternative explanation was offered in the review by Franceschi et al.(16) . Previously, in animal models, glucuronidase activity demonstrated in fecal content is indicative of enterohepatic circulation of iodothyronines via the gut microbiome, which allows reabsorption of native T3 following hydrolysis of conjugated forms of T3. The fraction of reabsorbed T3 that escapes liver extraction may re-enter the general circulation and contribute to the systemic pool of T3 (17) . In previous studies on centenarians, a longevity-specific gut microbiome remodeling and signature was observed in conjunctions with subtle differences in thyroid status, suggesting an association between the two(16). Based on these data the hypothesis can

be raised that increased reabsorption of T3 by the gut microbiome may contribute to the observed higher T3/T4 ratio in this study and previously published studies in centenarians.

It is tempting to speculate that the high circulating levels of TSH in offspring somehow contribute to their longevity phenotype. TSH receptors have been found in bone and adipose tissue, as well as in the thymus and the brain (18-22). The effects of TSH signalling on these tissues are not yet well understood; further research is necessary to increase our understanding of TSH's extrathyroidal role in physiology and aging.

The thyroid status and function in the oldest old are complex and very heterogeneous. This applies in particular for the physiological thyroid age-related changes that occur in the absence of overt clinically relevant pathologies. While previously regarded as simply detrimental, such changes are currently best conceptualized as part of the systemic, adaptive remodeling that helps humans survive in the last decades of human life. As such, these changes are intimately linked to other age-related changes such as those occurring in the gut microbiome and immune system, making it difficult to disentangle specific and unidirectional cause–effect relationships (16).

Our study had several strengths. All morning samples were taken with the participants in the fasted state, and all the other samples (throughout study day 1) were taken in between standardized meals, thereby minimizing variation in hormone levels due to gastrointestinal absorption or nutrition differences. The study consisted of a large number of time points across the study, thereby allowing detailed profiling of the T3, fT3 and TSH concentrations over time following the T3 challenge, allowing us to have observed any subtle differences in concentration profiles indicating polymorphism of the thyroid hormone receptor, deiodination enzymes or enzymes of the sulphating pathways. We are able to conclude with confidence that the T3 concentration profile and TSH decline are similar between offspring and controls, since this has been measured across 25 time points on study day 1 alone.

In conclusion, we found that, following a challenge with 100 µg triiodothyronine, offspring from long-lived families had similar T3, fT3 and TSH 96-h concentration profiles compared to similarly aged controls, indicating similar T3 turnover and similar negative T3 feedback on TSH. These findings, in combination with previously observed higher TSH levels and lower thyroid responsivity to TSH in members of long-lived families, suggest that the cause of high circulating TSH levels in familial longevity lies in the thyroid gland, although we cannot exclude concomitant dissimilar T4 feedback or increased T4 turnover. The role of TSH in the longevity phenotype remains a matter of speculation and requires further research.

Acknowledgments

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Data Availability

The datasets generated and analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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Chapter 6

Familial Longevity is Associated with Lower Baseline Bone Turnover but not Differences in Bone Turnover in Response to rhTSH. Ana Žutinić, Ferdinand Roelfsema, Hanno Pijl, Bart E. Ballieux, Rudi G.J. Westendorp, Gerard J. Blauw and Diana van Heemst.
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Abstract

CONTEXT. Offspring from long-lived families have a different thyroid status than controls, characterised by higher circulating levels of thyroid stimulating hormone (TSH) and similar levels of thyroid hormone. Expression of the TSH receptor has previously been observed on various extrathyroidal tissues, including bone. However, potential physiological consequences of differences in circulating TSH as observed in familial longevity on bone tissue remain unclear.

OBJECTIVE. Based on the hypothesis that TSH may inhibit bone resorption, we explored whether offspring of long-lived families have lower bone turnover than controls at baseline as well as following a challenge with recombinant human TSH (rhTSH).

METHODS. Bone turnover markers CTX and P1NP were measured in fasted morning samples from 14 offspring and 12 controls at baseline and at 24 hour intervals following 0.1mg rhTSH i.m. administration for four consecutive days.

RESULTS. At baseline, mean (SEM) CTX was 0.32 (0.03) ng/ml in offspring and 0.50 (0.04) ng/ml in controls, $p < 0.01$, whereas mean (SEM) P1NP was 39.6 (3.2) ng/ml in offspring and 61.8 (6.6) ng/ml in controls, $p < 0.01$. Following rhTSH administration, both CTX and P1NP levels transiently increased over time and normalized towards baseline after 72 h (general linear modelling: CTX time $p = 0.01$, P1NP time $p < 0.01$); the response was similar between offspring and controls.

CONCLUSIONS. Bone turnover markers were lower at baseline in offspring from long-lived families than in controls but increased similarly following an rhTSH challenge.

Introduction

Even subtle differences within the euthyroid range have been associated with exceptional human longevity and with differences in survival at old age[1-3]. Although the precise underlying mechanism of this observation is unknown, it could be due to the role that the hypothalamus-pituitary-thyroid axis plays in influencing numerous physiological processes and tissues across the different stages of the lifespan, such as energy metabolism and tissue maintenance and repair [4, 5].

In the Leiden Longevity Study (LLS)[6, 7], we have recently shown that offspring from long-lived families (offspring) have a different thyroid status than similarly-aged controls[8]. The offspring had consistently higher circulating thyroid stimulating hormone (TSH) concentrations, as measured every 10 minutes over a 24 hour period, in the absence of differences in thyroid hormone levels or TSH bioactivity[8]. To assess potential mechanisms that may cause this difference, we consequently performed two thyroid challenge studies in a subgroup of the same cohort: one challenge with recombinant human TSH (rhTSH)[9] and another with thyroid hormone triiodothyronine (T3) [10]. We found that offspring had a lower thyroidal responsivity to rhTSH stimulation than controls [9] and similar thyroid hormone T3 turnover [10].

The physiological consequences of this lower thyroidal responsivity to TSH as well as the higher circulating TSH levels in absence of differences in thyroid hormones in offspring, and how and whether these contribute to their predisposition for longevity, remain unknown. In addition to an established role in energy metabolism, it has been suggested that thyroid hormones play crucial roles in tissue maintenance and repair, which are of key importance to longevity [11]. Interestingly, expression of the TSH receptor has been observed on various extrathyroidal tissues, including the pituitary gland, thymus, testes, kidney, liver, adipose tissue, and bone. Based on these observations, it has been suggested that in addition to thyroid hormone, TSH may have a role in other tissues than the thyroid gland, including bone tissue[12-14].

Although tissue maintenance and repair are generally notoriously difficult to measure in humans [15], bone tissue turnover can be assessed reliably [16] through measurement of the serum osteoclastic bone resorption marker collagen type 1 C-terminal cross-linked telopeptide (CTX) and the osteoblastic bone formation marker N-terminal propeptide of type 1 collagen (P1NP).

In the case of bone, based on earlier studies, it was suggested that TSH inhibits osteoclastic bone resorption and that in addition to higher levels of thyroid hormone, low circulating TSH may contribute to bone loss [17-19]. Based on these observations, the hypothesis was formulated that the net result of slightly higher circulating TSH (while circulating levels of thyroid hormone are comparable), as observed in familial longevity, might be a slightly lower rate of bone resorption, which might protect against age-associated bone loss, and thus contribute to healthy longevity. The aim of the present study was to compare the levels of the bone markers CTX and P1NP in offspring from long-lived families and controls at baseline and following a rhTSH challenge over a four consecutive day study period. The specific objectives were threefold: i) to compare baseline levels of bone resorption marker CTX and bone formation marker P1NP between offspring and partners, ii) to explore whether circulating levels of these markers of bone resorption and bone formation would change after stimulation with rhTSH, and iii) to explore potential differences between offspring and partners in the response of these markers of bone resorption and bone formation after stimulation with rhTSH.

Results

Inclusions

The inclusion flow chart of subjects for the study is presented in Figure 1. In total, 30 subjects from the Leiden Longevity Study participated in the rhTSH study. One subject (male offspring) was excluded from bone marker analysis due to suspected i.v. rhTSH administration. Three subjects (female controls) were excluded from bone

marker analysis due to osteoporosis. Bone marker analysis was performed in 26 subjects (14 offspring and 12 controls).

Group characteristics

Baseline characteristics of the study population are presented in Table 1. Offspring and controls comprised an active, healthy, high middle-aged population and the groups were similar regarding age, sex, BMI and exercise level. Maternal age and paternal age were higher in offspring compared to controls ($p < 0.01$ and $p = 0.03$, respectively), in accordance with the longevity phenotype selection criteria. The distribution of males and females was similar between the offspring and control group ($p = 0.72$). Laboratory measurements of thyroid hormones and liver function were all within normal range and similar between offspring and controls, with baseline TSH being significantly higher in offspring than in partners ($p < 0.04$), a distinction characterising this cohort. Both groups had a mean kidney function within normal range ($\text{GFR} > 60 \text{ mL/min per } 1.73\text{m}^2$), which was similar between offspring and partners ($p = 0.11$). One participant, a female offspring, had GFR 54 at screening, but was included in the study due to absence of any indication of chronic (kidney) disease.

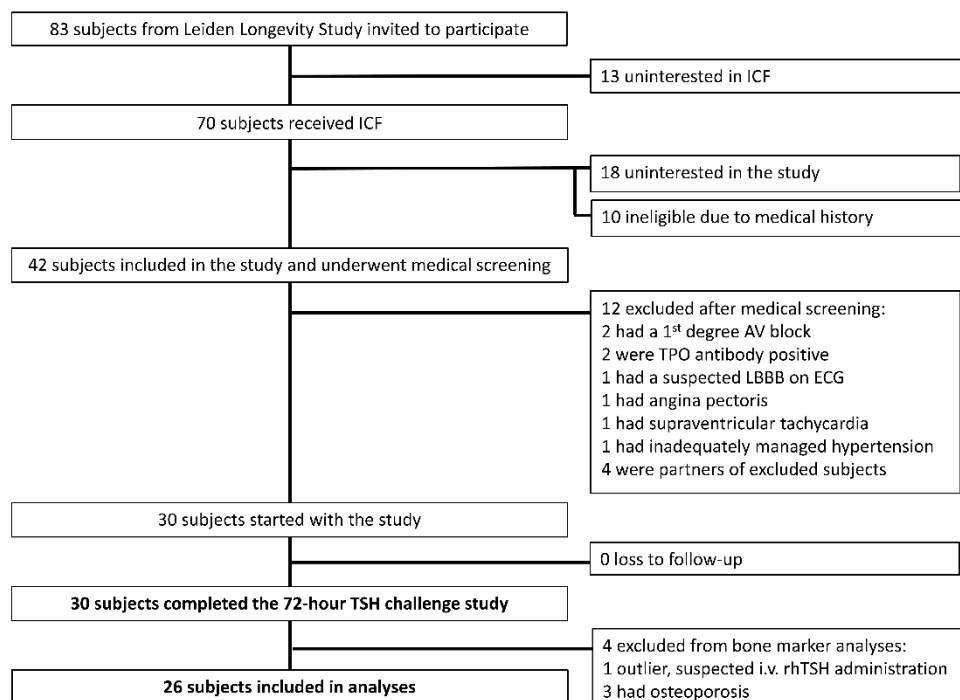


Figure 1. Inclusion flow chart bone marker analysis in the recombinant human thyroid stimulating hormone (rhTSH) study. ICF: informed consent form, AV: atrioventricular, TPO: thyroid peroxidase, LBBB : left bundle branch block, ECG: electrocardiogram, i.v.: intravenous.

Table 1. Baseline characteristics of the study population.

	Offspring (n=14)	Controls (n=12)	P value
Demographics			
Age mother <i>years</i> *	93 (91–97)	77 (69–90)	<0.01
Age father <i>years</i> *	93 (73–96)	78 (64–86)	0.03
Male <i>n (%)</i>	8 (57)	6 (50)	0.72
Age <i>years</i>	69 (5)	68 (7)	0.87
Anthropometrics			
BMI <i>kg/m²</i>	25.8 (4.3)	26.1 (3.3)	0.84
Height <i>cm</i>	173.7 (10.9)	173.6 (9.0)	0.97
Fat mass <i>kg</i>	23.9 (7.2)	25.2 (5.8)	0.62
Lean mass <i>kg</i>	54.1 (12.9)	53.0 (13.3)	0.83
Vitamins and exercise			
Vitamin D	1 (7)	1 (8)	0.27
supplementation <i>n (%)</i>	12 (86)	9 (75)	0.49
Regular weekly exercise	4 (29)	4 (33)	0.80
<i>n (%)</i>	8 (57)	5 (42)	0.44
- Low intensity (walking)			
<i>n (%)</i>			
-High intensity (jogging,			
tennis) <i>n (%)</i>			
Laboratory			
measurements	71.2 (13.9)	78.9 (8.6)	0.11
GFR <i>ml/min per 1.73m²</i>	22.3 (4.1)	25.3 (7.4)	0.20
AST <i>U/L</i>	19.6 (5.3)	21.7 (5.0)	0.32
ALT <i>U/L</i>	3.3 (1.7)	2.07 (1.0)	0.04
Baseline TSH <i>mU/L</i>	13.9 (13.0–15.8)	15.3 (14.4–15.7)	0.11
Baseline fT4* <i>pmol/L</i>			

All values are mean (standard deviation) unless otherwise stated. BMI: body mass index, GFR: glomerular filtration rate, AST: aspartate transaminase, ALT: alanine transaminase, TSH: thyroid stimulating hormone, fT4: free T4, fat mass: fat mass, in kilograms, as measured by Bioelectrical Impedance Analysis, lean mass: fat free mass, in kilograms, as measured by Bioelectrical Impedance Analysis.*Median (interquartile range).

Baseline bone markers

Baseline bone markers in offspring and controls are shown in Figure 2. Bone resorption marker CTX was significantly lower in offspring than in controls (mean (SEM) 0.32 (0.03) ng/ml and 0.50 (0.04) ng/ml, respectively, $p < 0.01$). The same was observed for the bone formation marker P1NP, which was also significantly lower in offspring than in controls (mean (SEM) 39.6 (3.2) ng/ml and 61.8 (6.61) ng/ml, respectively, $p < 0.01$).

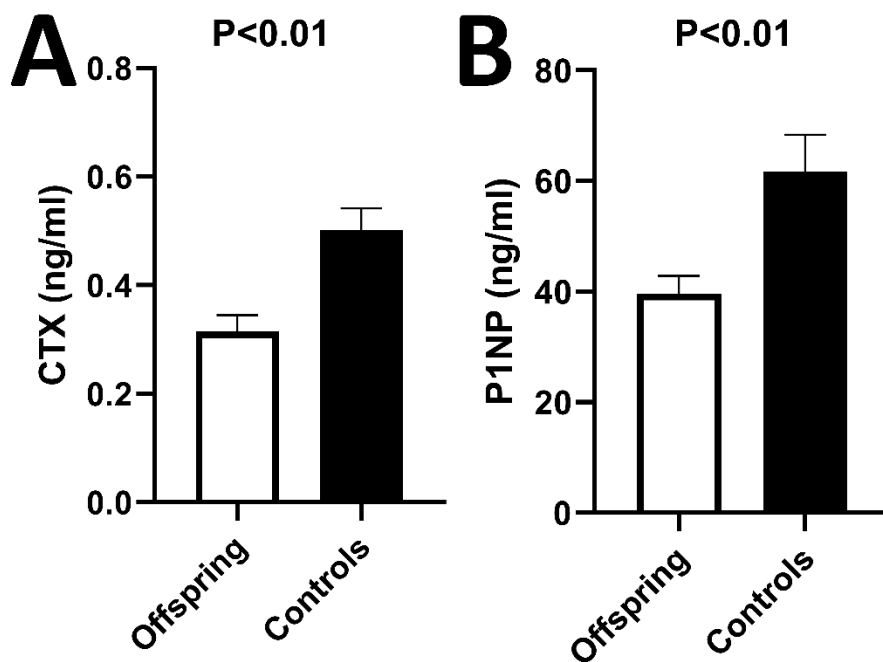


Figure 2. Bone resorption (CTX) and bone formation (P1NP) markers in offspring versus controls at baseline of rhTSH study. A) Mean CTX at baseline of rhTSH study in offspring (n=14) and controls (n=12), p value<0.01. B) Mean P1NP at baseline of rhTSH study in offspring (n=14) and controls (n=12), p value<0.01. rhTSH: recombinant human thyroid stimulating hormone, CTX: collagen type 1 C-terminal cross-linked telopeptide (CTX), P1NP: N-terminal propeptide of type 1 collagen. Error bars: standard error of the mean. P value < 0.05 was considered statistically significant.

The bone markers were not different between men and women (CTX: mean (SEM) 0.36 (0.03) ng/ml and 0.45 (0.06) ng/ml respectively, p=0.16; P1NP: mean (SEM) 42.8 (2.8) ng/ml and 58.2 (7.8) ng/ml respectively, p=0.06). Both male and female offspring had lower bone markers than male and female controls. Within males, P1NP

was significantly lower in offspring than in controls (mean (SEM) 36.7 (3.0) and 50.8 (3.0) ng/ml in offspring and controls respectively, $p<0.01$), while CTX followed a similar trend (mean (SEM) 0.31 (0.04) and 0.42 (0.04) ng/ml in offspring and controls respectively, $p=0.10$). Within females, CTX was lower in offspring than controls (mean (SEM) 0.32 (0.05) and 0.58 (0.08) ng/ml in offspring and controls respectively, $p=0.01$), and P1NP followed the same trend (mean (SEM) 43.5 (6.6) and 72.8 (11.7) ng/ml in offspring and controls respectively, $p=0.05$).

Bone markers following rhTSH administration

The concentration profiles of bone markers CTX and P1NP over time following rhTSH administration are shown in Figure 3. Upon visual inspection, the response in bone markers to rhTSH administration seemed similar in offspring and controls. Both CTX and P1NP markers increased following rhTSH administration (CTX ANOVA=0.01, P1NP ANOVA<0.01, Table 2), with CTX displaying the highest increase 48 hours after administration while P1NP reached highest value at 24 hours following rhTSH administration. Bone markers normalized towards baseline at 72 hours following rhTSH administration.

Despite the increase in bone markers following rhTSH administration, concentration of both CTX and P1NP remained lower in offspring than in controls throughout the study (CTX: $p=0.01$, 0.01 and 0.01 at 24, 48 and 72 hours, respectively; P1NP: $p=<0.01$, <0.01 and <0.01 at 24, 48 and 72 hours, respectively).

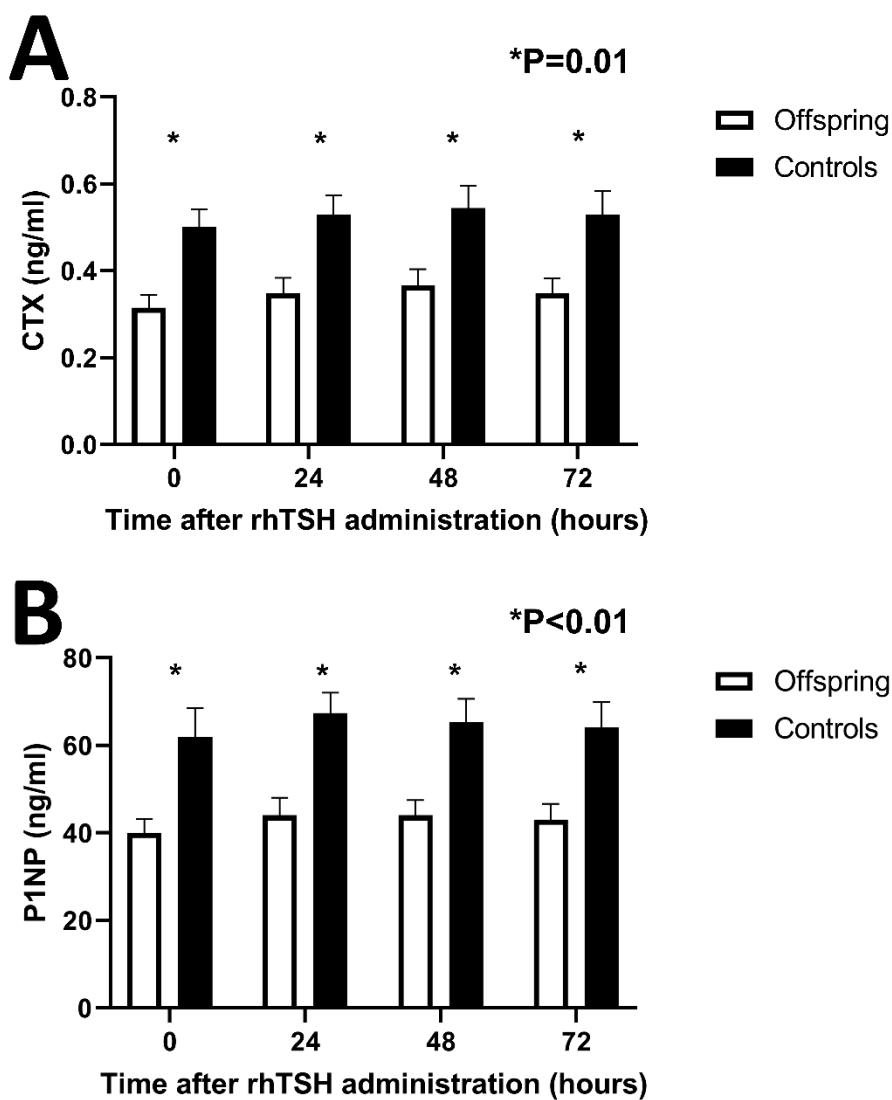


Figure 3. Concentration profiles of bone resorption (CTX) and bone formation (P1NP) markers over time in offspring from long-lived families (n=14) and controls (n=12) following a challenge with 0.1mg rhTSH. A) Mean circulating CTX at 24-hour intervals following rhTSH administration. B) Mean circulating P1NP at 24-hour intervals following rhTSH administration. rhTSH: recombinant human thyroid

stimulating hormone, CTX: collagen type 1 C-terminal cross-linked telopeptide (CTX), P1NP: N-terminal propeptide of type 1 collagen. Error bars: standard error of the mean.

Testing for interaction using general linear modelling confirmed that the effect of rhTSH administration on bone markers was not statistically different between offspring and controls, nor between males and females (Table 2).

Table 2. Assessment of effects of time, and its interaction with group and gender using general linear model analysis of bone resorption and formation markers following recombinant human TSH administration in 26 study subjects (14 offspring and 12 controls).

	Bone resorption (CTX)	Bone formation (P1NP)
Within		
Time	P=0.01	p<0.01
Time*group	p=0.95	p=0.85
Time*gender	p=0.14	p=0.95

Discussion

Based on the hypothesis that TSH may inhibit bone resorption, the specific objectives of this study were i) to compare baseline levels of bone resorption marker CTX and bone formation marker P1NP between offspring and controls, ii) to explore whether circulating levels of these markers of bone resorption and bone formation would change after stimulation with rhTSH, and iii) to explore potential differences between

offspring and controls in the response of these markers of bone resorption and bone formation after stimulation with rhTSH.

The novel findings of this study are threefold. Firstly, familial longevity is characterized by lower bone turnover at baseline, indicating that bone tissue is yet another organ system possibly impacted by the longevity phenotype. Secondly, rhTSH administration increases bone turnover markers, indicating that exogenous TSH and/or endogenous thyroid hormones are drivers of bone turnover. Finally, the response to rhTSH administration in bone markers was similar in members of long-lived families as controls, indicating that, at least in bone tissue, offspring and controls respond similarly to this challenge of the thyroid axis. The differences between offspring from long-lived families and controls in maintenance and repair of other tissues, as well as the many variables regarding the specific and direct influence of TSH, fT4 and fT3 on bone turnover markers, remain to be elucidated in further research.

The baseline differences in bone markers between offspring and controls are in line with briefly mentioned findings from a previous subgroup of the Leiden Longevity Study cohort[20] and could be indicative of a generally lower rate of tissue turnover that might contribute to the offspring's longevity phenotype. Further studies into maintenance and repair of other tissues could provide greater insights whether a lower rate of tissue turnover is a generalized phenomenon in offspring from long-lived families compared to controls, or whether this effect is specific to the bone. It remains subject to speculation whether the difference in thyroid status plays a role in the underlying mechanism (7).

Following rhTSH administration, in this novel and explorative study on the influence of a thyroid axis challenge on markers of bone turnover in a healthy, high middle-aged population, we show that a single dose of 0.1mg rhTSH transiently increased bone turnover.

Previous studies report conflicting findings when it comes to direct effects of TSH on bone turnover, from a positive to a negative to no association, although most studies report a negative association between TSH and bone remodelling [12, 21]. The TSH receptor (TSHR) has been identified in osteoclasts and osteoblasts, but at such low levels that a physiological role of TSH on bone tissue was deemed unlikely[22]. It could be that a challenge situation with rhTSH was able to adequately trigger the TSHR to influence bone tissue despite the low levels of its expression in bone cells[21].

Still, in our study, we are not able to disentangle the influence of TSH from the influence of thyroid hormones on bone turnover markers, since we studied healthy offspring and controls, as opposed to subjects who have undergone a thyroidectomy (in which the direct effect of rhTSH on bone turnover markers could be measured). In participants with an intact thyroid gland, conclusions about TSHR responsivity or direct effects of TSH on bone tissue cannot be drawn because any effect of rhTSH cannot be disentangled from the effect of thyroid hormones.

We do show that rhTSH administration, which is a condition of increasing exogenous circulating TSH and increasing endogenous circulating thyroid hormone levels, is able to transiently increase bone turnover. Moreover, following rhTSH administration, it is mostly levels of thyroid hormone fT4 that are increased[9], and our findings here support the role of thyroid axis, whether through TSH or thyroid hormone, in increasing bone turnover. In future studies, we aim to investigate bone turnover following a challenge with thyroid hormones specifically.

Despite our previous observation of a difference in thyroid responsivity to rhTSH between offspring and controls[9], we did not find a difference in their bone markers' responses to rhTSH. This could be due to slight increases in thyroid hormone levels already being sufficient in maximally increasing bone markers, therefore even lower thyroidal responsivity to rhTSH in offspring might already be adequate in maximally

increasing bone marker concentrations. The influence of thyroid hormones on bone marker concentrations remains our target for future research.

Our study had several strengths. All samples were fasted and obtained in the mornings at 24 h intervals which means that our measurements were minimally disturbed by circadian rhythm of the bone markers or meal variation, known to especially influence CTX to a large degree[20].

This study also has a number of limitations. The main limitation of our study concerns the study design, which was not primarily established for measurement of bone markers. This creates uncertainty regarding the sample size as well as regarding other factors besides the thyroid axis that might influence bone tissue turnover, such as physical stress, steroids, parathyroid hormone, calcitonin and growth hormone (GH) – information on which was not obtained during the rhTSH study and could not be accounted for in our analyses.

Although the TSH challenge study has been powered on the analysis of the primary endpoint, the analyses of secondary endpoints as presented in this paper do represent preconceived explorative analyses. The value of this study as compared to other studies in healthy humans which are observational in design, is that we were able to study the time effect of transient changes in thyroid status in two groups of healthy humans, a group enriched for familial longevity and a control group. In future research, we suggest further investigation in a larger sample of the Leiden Longevity Study of bone phenotypes (using imaging techniques) and including measurements of serum concentrations of calcium, cholecalciferol and metabolites, calcitonin, parathyroid hormone, cytokines and IGF-I, which all impact on bone metabolism. Previously, GH, which also impacts bone metabolism was shown to be different in familial longevity [23, 24].

Another limitation is the existence of uncertainty regarding the selection of familial longevity. By design of the Leiden Longevity Study, the offspring are derived from long-lived families, while controls are the partners of the offspring from long-lived

families. Although this design does allow for a comparison between (offspring and control) groups that are matched for age and important adult environmental factors such as socio economic status, a limitation of this design is that contrast between groups may be diluted, as some offspring may be less likely than others to have inherited a favourable genetic predisposition, while some controls may actually also have long-lived parents. Despite this limitation, maternal age and paternal age were higher in offspring compared to controls in accordance with the longevity phenotype selection criteria. To maximise the contrast in familial longevity between groups, future studies should focus on the use of scores which include family longevity history information for both offspring and controls, such as Longevity Relatives Count scores [25].

Methods

Study participants

The LLS was founded in 2002 and designed to investigate genotypes and phenotypes of long-living families (men aged 89 and older, women aged 91 and older) living in The Netherlands in early 2000s [6], without any restrictions on health or demographics[7]. The offspring of these families were asked to participate in the study, with their current partners as controls, thereby creating a case group enriched for longevity and a control group with similar lifestyle factors and socio-economic status, but without selection for familial predisposition to longevity.

Recruitment and study details regarding the study have been described elsewhere [9, 10]. In brief, offspring and controls from the LLS subgroup previously studied in terms of thyroidal status[8] were recruited, with the following exclusion criteria: laboratory results (haemoglobin < 7.1 mmol/L, TSH > 4.0 mU/L, fT4 < 9 pmol/L or > 24 pmol/L, TPO antibody positivity (>35 kU/L)), medical history (cardiac arrhythmias, (history of) thyroid diseases, renal, hepatic or endocrine disease, or any

other significant chronic disease), medication use (hormone therapy, thyroid medication), lifestyle factors (nicotine abuse, (history of) alcohol abuse (>28 units per week)) and other factors (difficulty inserting an intravenous cannula, participation in other research projects within the last 3 months, participation in two or more projects in one year, evaluation by a physician as too frail or vulnerable to participate).

Study protocol

Participants were admitted into the study after passing medical screening. The rhTSH study consisted of four consecutive study days at Leiden University Medical Centre. On the morning of study day 1, an intravenous cannula was placed in a forearm vein, blood was withdrawn at baseline and rhTSH was administered through intramuscular injection (recombinant human TSH, 0.1 mg/mL in 1 mL, gluteal muscle). The time of injection was used as reference, time zero. On study day 2, 3 and 4, fasted blood samples were obtained at respectively 24, 48 and 72 h after rhTSH injection.

During study day 1, subjects were mostly sedentary in the study room and received standardized meals (Nutridrink Compact, Nutricia Advanced Medical Nutrition, Zoetermeer, The Netherlands). During other study days, subjects visited the LUMC in the morning and were at their leisure the rest of the day; meals were not standardized.

Height, weight and body composition were measured on day 2. Body composition was measured with a Bioelectrical Impedance Analysis meter at a fixed frequency of 50kHz (Bodystat 1500 Ltd, Isle of Man, British Isles[26]). Exercise level was determined by a questionnaire about type of exercise, duration and weekly frequency.

The studies were designed in accordance with the declaration of Helsinki and have been approved by the Medical Ethical Committee of the Leiden University Medical Centre. They are registered at Leiden University Medical Centre under the protocol P16.107 and with EudraCT under the number 2016-001497-15. All subjects gave written informed consent prior to medical screening.

Handling of samples

Serum samples were kept at room temperature for 60 min to clot before processing at the Department of Clinical Chemistry and Laboratory Medicine, Leiden University Medical Centre, The Netherlands. Samples were centrifuged for 10 min at 2350 G relative centrifugal force at a temperature of 20 degrees Celsius. After being transferred to 500 microliter aliquots, serum samples were temporarily stored at –20 degrees Celsius prior to permanent storage at –80 degrees Celsius until analysis.

Laboratory measurements

Laboratory measurements in serum samples were performed after all subjects had completed the study. All measurements were performed with the same lot number. For each participant, samples from the different time points were measured in the same batch.

Assays and assay performance

All measurements were performed with fully automated, software monitored equipment and diagnostics from Roche Diagnostics (Almere, The Netherlands) at the Department of Clinical Chemistry and Laboratory Medicine at Leiden University Medical Centre, The Netherlands. Aspartate aminotransferase (AST) (Catalogue number 11876848216), alanine aminotransferase (ALT) (Catalogue number 11876805216) and creatine (Catalogue number 5168589190) for estimating glomerular filtration rate (GFR) were measured from a fasted morning serum sample using the Modular P800 clinical chemistry analyser. GFR was calculated using the CKD-EPI calculation[27]. Thyroid parameters TSH (Catalogue number 11731459122, research reference identifier (RRID): AB_2756377), fT4 (Catalogue number 6437281190, RRID: AB_2801661), T4 (Catalogue number 12017709122, RRID: AB_2756378), fT3 (Catalogue number 6437206190, RRID: AB_2827368) and T3 (Catalogue number 11731360122, RRID: AB_2827369), and bone markers (CTX and P1NP) were measured in serum by an immunoassay using Roche cobas8000 with an E602 module. The coefficients of variation (CV) were 2.33 (SD 0.01) for CTX, 2.28 (SD 0.77) for P1NP, 2.36 (SD 0.52) for TSH and 5.55 (SD 2.28) for fT4.

Statistical analysis

Descriptive statistics were used to summarise group characteristics. Independent samples T test, Mann-Whitney U test and Chi square test were used, depending on the characteristics of the variable (normally distributed, not normally distributed and categorical, respectively), to statistically test for differences between offspring and controls regarding baseline characteristics. General linear modelling (GLM) was used for repeat measurements (analysis of variance, ANOVA) to investigate the effects of time and sex on the concentration profiles of CTX and P1NP, and the interaction term time* (offspring/control) status was used to test for differences in time dependent changes in CTX and P1NP between offspring and controls. In all analyses, $P \leq 0.05$ was considered statistically significant.

Programs used for statistical analyses were SPSS for Windows, version 23 (SPSS, Chicago, IL), Systat version 13 (Systat Software, Inc, San Jose, CA) and Matlab (The MathWorks Inc, Natick, MA). Graphs were made using Microsoft Office Excel 2016 and GraphPad Prism for Windows, version 8.1.1 (330) (GraphPad Software, Inc, San Diego, CA).

Abbreviations

ANOVA	Analysis of variance
ALT	Alanine transaminase
AST	Aspartate transaminase
BMI	Body mass index
CTX	Collagen type 1 C-terminal cross-linked telopeptide
fT4	Free thyroxine
fT3	Free triiodothyronine
GFR	Glomerular filtration rate
GH	Growth hormone
GLM	General linear modelling
LLS	Leiden Longevity Study
P1NP	N-terminal propeptide of type 1 collagen
rhTSH	Recombinant human thyroid stimulating hormone
RRID	Research reference identifier
T3	Triiodothyronine
T4	Thyroxine
Tg	Thyroglobulin
TPO	Thyroid peroxidase
TSH	Thyroid stimulating hormone

Author contributions

Ana Zutinic: conceptualization, investigation, formal analysis, writing – original draft, supervision. Ferdinand Roelfsema: conceptualization, formal analysis, writing – review & editing. Hanno Pijl: conceptualization, writing – review & editing. Bart E. Ballieux: resources, writing – review & editing. Rudi G.J. Westendorp: conceptualization, writing – review & editing. Gerard J. Blauw: writing – review & editing. Diana van Heemst: funding acquisition, conceptualization, supervision, writing – review & editing.

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Conflict of interest

The authors have no conflicts of interest to declare.

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Chapter 7

General discussion and future perspectives

As the fraction of the older population increases, and with it the prevalence of chronic diseases and costs of health care, great effort is being made to understand healthy ageing. The thyroid axis plays an essential role in multiple physiologic processes and has been vastly implicated in ageing (1), making it one of the prime candidates of research and candidates for innovation.

The Leiden Longevity Study (LLS) (2) is an established cohort for investigating differences between offspring of long-lived families and controls of similar age and socio-economic background, and previous studies among LLS participants showed that families with a lower mortality history score had the highest thyroid stimulating hormone (TSH) and lower thyroid hormone (TH) levels (3). When the thyroid axis in LLS offspring and controls was investigated in more detail through a series of measurements every 10 minutes over a period of 24 hours, it was found that the circulating TSH levels were consistently higher in offspring than in controls (4). Interestingly, throughout the whole study period, the circulating TH levels were not higher but were comparable between offspring and controls (4). Theoretically, this finding could be explained by a lower TSH bioactivity in offspring. However, the TSH bioactivity was investigated and found not to be different between offspring and controls (4). Two other possible underlying mechanisms were formulated that were fundamental for the work described in this thesis, namely that:

- i) Offspring from long-lived families might have lower responsivity of the thyroid gland to stimulation by TSH, thereby needing higher levels of circulating TSH for the same TH secretion,
- and/or
- ii) Offspring from long-lived families might have higher turnover of TH in the circulation, thereby requiring higher circulating TSH for adequate circulating TH levels.

Through two challenge studies, one with 0.1mg recombinant human TSH (rhTSH) in 30 LLS participants, and one with 100mcg synthetic triiodothyronine (T3) in 27 LLS participants, we found that offspring have lower thyroidal responsivity than controls, and that offspring have similar TH concentrations and T3 feedback on TSH levels. These findings support the first hypothesis, dismiss the second hypothesis, and make the combination of the two superfluous.

These findings indicate that the mechanism behind the different thyroid status in familial longevity found among the offspring of LLS lies in the responsivity to TSH of the thyroid gland. Although the precise mechanism causing the difference in TSH responsivity remains unknown, this could be related to the TSH receptor (TSH-R) rather than to a difference in the metabolism of TH.

The full study design used to investigate these hypotheses is outlined in

Chapter 2. The inclusion of participants shows that almost two thirds of the initially approached participants were not eligible for the study, underlining the difficulty of performing intervention studies in a healthy, older population even in a dedicated cohort, due to high prevalence of chronic diseases, especially cardiovascular disease, in older age. Significant cardiovascular disease as well as other significant comorbidities were one of the main exclusion criteria for our study in order to minimise the risk of adverse events such as atrial fibrillation upon administration of rhTSH or TH. In future challenge studies with LLS participants, caution should be employed to prevent selection bias; due to the fact that morbidity is more prevalent among controls(2), selecting only healthy participants could lead to the selection of the healthiest controls, who might most resemble offspring in their phenotype, and not be truly representative of the control population, thus diluting potential differences between groups.

In **Chapter 3** we determined the optimal frequency of thyroid parameter measurements in a healthy, older population following a challenge with 0.1mg i.m. rhTSH by measuring samples from all 17 study time points in six participants. We

show that in the first 8 hours following 0.1mg i.m. rhTSH administration, TSH concentrations vary at relatively high frequency and may best be measured often (every 15 minutes), while measurement of TH is adequate at lower frequency of every two hours. All parameters may be measured at 24, 48 and 72 hours following rhTSH administration to observe their return to baseline. Thyroglobulin (Tg) displayed very low variability in the first 8 hours, and may be measured at baseline, and then 24 hour intervals following rhTSH administration. The measurement points suggested are comparable to those previously used in healthy younger and middle-aged populations, where parameters were measured at 2-4 hours intervals and for up to 72 hours(5,6). In future studies, as an addition to our time points, we advise repeated baseline measurements for calculation of mean baseline hormone levels.

The primary outcomes of the rhTSH challenge as measured in the whole cohort are outlined in **Chapter 4**. Following intramuscular administration of 0.1mg rhTSH, the ratio of area under the curve (AUC) of free T4 to AUC of TSH (AUC fT4/AUC TSH) as well as AUC Tg/AUC TSH ratio were lower in offspring than in controls. These novel findings indicate that offspring have lower thyroidal responsivity to TSH than controls.

The primary outcomes of the T3 challenge as measured in the whole cohort are outlined in **Chapter 5**. In a healthy, older population, following a challenge with 100mcg T3, offspring and controls had similar circulating T3 levels and similar circulating TSH levels, indicating similar turnover of T3 and similar negative feedback on TSH, respectively. In future studies, labelled T3 could be administered in a smaller sub group to indeed confirm similar turnover between offspring and controls.

In **Chapter 6**, exploratory measurements are reported of bone metabolism in offspring from long-lived families and controls at baseline and following the rhTSH challenge. TSH receptor has been identified on bone cells and is likely to enhance bone resorption. In our study, following the rhTSH challenge, the increase in bone marker levels was not different between offspring and controls. However, baseline

levels of circulating bone markers CTX and P1NP were lower in offspring than in controls, as was previously briefly shown in Switchbox study. This could be due to a preserved regenerative capacity in offspring compared to controls, in this case of bone tissue. Interestingly, our findings in bone markers following rhTSH administration do not show a clear difference in direct effects of TSH on bone tissue between offspring and controls, as could have been the case with differences in the TSH receptor. It is possible that any effect of the TSH receptor has been confounded by also increased circulating thyroid hormones. Unfortunately, in a study of a healthy human population it is not possible to discern increasing TSH from increasing TH. However, by performing bone marker measurements following a rhTSH challenge in a population of patients with thyroidectomy, direct effects of TSH on bone markers, regardless of TH, could be measured. It is not clear whether these patients also have variations of the TSH receptor.

In this thesis, for the first time, we describe in detail the circulating TSH and TH levels in a healthy, euthyroid, older population following 0.1mg i.m. rhTSH administration and also following 100 mcg oral T3 administration. The two challenge studies performed are complementary: one investigating the feed forward mechanism and one investigating the feedback mechanism of the thyroid axis in offspring of long-lived families and controls. The conclusions drawn align with results of previous work related to thyroid function and ageing in larger populations. In the Leiden 85 study, with 599 participants aged 85 years and followed-up for four years, higher circulating TSH has not been associated with adverse effects and has been associated with lower mortality rate(7). Moreover, in centenarians, there is an increased prevalence of higher circulating TSH(8), indicating its possible beneficial effects on longevity. A randomized controlled trial in an older population with subclinical hypothyroidism, where circulating TSH is elevated but TH are within euthyroid range, showed that treatment with TH levothyroxine is not beneficial compared to placebo(9).

The results of the two thyroid axis challenge studies for the first time implicate lower thyroidal responsivity to TSH as the mechanistic underpinning for the previously

found higher circulating TSH in the absence of differences in TH between offspring from long-lived families and controls(4). These findings indicate the likely importance of TSH and/or the TSH receptor in longevity. It is possible that the lower TSH receptor responsivity in offspring results in higher circulating TSH levels necessary for adequate TH release in offspring, and that this elevated, albeit within euthyroid range, circulating TSH carries longevity enhancing benefits. The TSH itself might have an effect on the activity of deiodinase enzymes in tissues and thereby regulate tissue specific TH availability. Through these effects, TSH could indirectly play an important role in local maintenance and repair processes, and thereby influence the chance of developing age-related diseases. Although tissue-specific TH levels are challenging (if not impossible) to manipulate in humans, animal models can be used to study tissue-specific inactivity or overexpression of TH and its effects on development of tissue-specific disease. Similar work has been performed by THYRAGE partners in bone, muscle and brain tissue(10-12). In the future, these studies could contribute to using TSH as means of tissue-specific delivery of TH.

Moreover, the TSH receptor has recently been identified on both bone tissue as well as in the thymus(13,14). Further investigation of the TSH receptor as well as TSH specific effects on tissues other than the thyroid are necessary. In future studies, thyroid biopsy in offspring from long-lived families and controls and TSH receptor genotyping could help identify differences between the TSH receptors in the two groups.

This thesis explored the primary outcomes of the thyroid axis challenge studies. However, direct effects of TSH on tissues other than the thyroid, as well as the secondary objectives of both challenge studies (such as cardiovascular effects, metabolomics and immunological factors such as the effect on peripheral blood mononuclear cells), remain to be investigated.

It is also possible that other hormonal axes, which influence the thyroid axis, are indirectly implicated in the differences in TSH status found between offspring and

controls. For example, dopamine regulates TSH secretion and could be lower in familial longevity, thereby contributing to higher circulating TSH levels. The longevity benefits attributed to higher circulating TSH could in part be due to lower dopamine levels. To test this hypothesis, future studies should measure circulating dopamine in relation to familial longevity, possibly in the study participants investigated in this thesis, or in other (healthy, older) populations with higher circulating TSH.

Identifying higher circulating TSH as implicated in familial longevity might also be of value for clinical practice, since higher circulating TSH might be of therapeutic importance. In animal studies, it has been shown that TSH administration can improve bone health by preventing and restoring bone loss(15). Since recombinant human TSH can be administered intramuscularly and intravenously in humans, and we show here that it does not cause adverse events at a low dose in a healthy older population, it may be useful for treating bone loss, for example in post-menopausal women under risk for developing osteoporosis, but also as a long-term supplement for enhancing healthy ageing. Of course, further studies are needed to investigate the benefits, length of intervention, number needed to treat and side-effects of such a treatment.

Healthy ageing remains a complex and nuanced subject not easily answered with a one-for-all solution(16). This thesis emphasizes the notion that the thyroid axis is vastly implicated in longevity and healthy ageing, through a great role of TSH and thyroid gland responsivity to TSH, possibly through the TSH receptor. Through this work of studying healthy ageing and deciphering its underlying mechanisms, we advance understanding of healthy ageing, and thereby strive to facilitate innovation in the field that will benefit individuals as well as society in the coming decades.

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Chapter 8

English summary

This thesis covers the study design, primary outcomes and first secondary outcomes of the TSH and T3 challenges in participants from the Leiden Longevity Study (LLS).

In **Chapter 1** we emphasize the importance of understanding the mechanisms underlying healthy ageing, due to an increasing size of the older demographic locally as well as globally, and with it the corresponding rise of prevalence of age-related diseases. Moreover, we outline the impact of the thyroid axis on physiological processes and its potential role in healthy ageing.

In **Chapter 2** we report the study design and data collected throughout the TSH and T3 study. In total, there were 17 blood withdrawal moments in TSH study, among 30 participants, and 25 blood withdrawal moments in T3 study, among 27 participants. Other data such as peripheral blood mononuclear cells, continuous physiological monitoring, questionnaires and biobank samples were also obtained for further research.

Chapter 3 investigates and reports the results of a pilot study where thyroid parameters were determined at all 17 blood withdrawal moments during TSH study. Optimal measurements times are identified for a healthy, older population, with mean TSH concentration showing the greatest variation in the first 8 hours following rhTSH administration, mean T4, fT4, T3 and fT3 showing variation from 2 hours after rhTSH administration, and mean Tg only showing variation at later time points, namely 24, 48 and 72 hours after rhTSH administration.

Chapter 4 reports the primary outcomes of the TSH study: the TSH and thyroid hormone profiles following administration of 0.1mg i.m. rhTSH in offspring and controls from LLS. The area under the curve (AUC) fT4/AUC TSH ratio was significantly lower in offspring than in controls (estimated mean (95%CI) 1.6 (1.2-1.9) and 2.2 (1.9-2.6), respectively, $p=0.01$), as was the AUC Tg/AUC TSH ratio (median (IQR) 2.1 (1.4-3.6) and 3.2 (2.7-7.4), respectively, $p=0.04$). We observed the same trend with the AUC fT3/AUC TSH ratio, although the difference was not statistically significant (estimated mean (95%CI) 0.6 (0.4-0.7) and 0.7 (0.6-0.8), respectively,

$p=0.07$). Overall, we show that members of long-living families have a lower thyroid responsivity to TSH compared to controls.

Chapter 5 reports the primary outcomes of the T3 study: the thyroid hormone and TSH profiles following administration of 100mcg T3 in offspring and controls from LLS. The concentration of thyroid hormones was similar in offspring and controls following the challenge (T3 GLM $p=0.11$, fT3 GLM $p=0.46$), and the feedback on TSH was similar also (GLM $p=0.08$), as was the relative TSH decline (%). indicating that there are no apparent differences in thyroid hormone turnover between offspring and controls.

In **Chapter 6**, the first secondary outcomes of the TSH and T3 challenge are investigated by determining bone formation markers (P1NP) and bone resorption markers (CTX) in offspring and controls. We found that bone turnover markers were lower at baseline in offspring from long-lived families than in controls ($p<0.01$ for both) but that they increased similarly following an rhTSH challenge in both groups.

In **Chapter 7**, we reflect on our findings and suggest further research and innovation on TSH, the TSH receptor and other hormonal axes influencing the thyroid axis, as possibly important for differences in longevity and healthy aging between offspring and controls.

Chapter 9

Appendices

Dutch summary (Nederlandse samenvatting)

Veroudering en vergrijzing

Met de steeds ouder wordende samenleving (1) zijn veroudering en vergrijzing niet alleen in Nederland, maar wereldwijd een actueel onderwerp. Naar verwachting zal het aantal mensen van 60 jaar en ouder wereldwijd meer dan verdubbelen tegen 2050, naar een aantal van 2.1 miljard mensen in vergelijking met 962 miljoen in 2017 (2). Tegelijkertijd wordt wereldwijd ook een verdrievoudiging van het aantal mensen van 80 jaar en ouder verwacht in dezelfde periode, naar 425 miljoen van 137 miljoen in 2017(2). In Nederland wordt tegen 2050 een ruime stijging verwacht in het percentage van de bevolking van 65 jaar en ouder ten opzichte van 2015, net als een stijging van het percentage van de bevolking van 80 jaar en ouder (3). Daarmee gaat de groeps grootte van mensen van 85 jaar en ouder, ‘de oudste ouderen’, ook groeien.

Veroudering is de belangrijkste risico factor voor het ontwikkelen van chronische ziekte, en de hiermee gepaard gaande functionele achteruitgang (4). Dit maakt onderzoek naar factoren die aan gezond oud worden bijdragen en die ten grondslag liggen aan het verouderingsproces zeer waardevol en niet alleen belangrijk voor het individu, maar ook voor de duurzaamheid van de gezondheidszorg en de maatschappij - op lokaal maar ook op globaal niveau.

Schildklierhormoon

Een van de hormonen die een rol speelt in gezond oud worden, is het schildklierhormoon. De beschikbaarheid van schildklierhormoon wordt door middel van een complex netwerk nauwkeurig gereguleerd, aangezien het vele noodzakelijke fysiologische processen in het lichaam beïnvloedt – van groei en ontwikkeling, tot stemming en cognitie, en ook immuniteit en het metabolisme (5,6).

Feedforward en feedback regulatie

Het constant houden van de spiegels van schildklierhormoon in het bloed wordt geregeld door een samenspel van hormonen die elkaar beïnvloeden via feed forward (stimuleren van secretie) en feedback (remming van secretie) signalen. Dit begint in de

hersenen, waar in de hypothalamus de thyrotrophin releasing hormoon (TRH) wordt uitgescheiden om de hypofyse te stimuleren tot productie en secretie van de schildklier stimulerend hormoon (TSH). TSH stimuleert de schildklier, door binding aan de TSH receptoren (TSHR), om schildklierhormonen te produceren en in het bloedsomloop uit te scheiden. De schildklier produceert ook thyroglobuline (Tg), een voorloper van de schildklierhormonen, waaruit weer het inactieve schildklierhormoon thyroxine (T4) en ook de actieve hormoon, triiodothyronine (T3) worden gegenereerd en uitgescheiden. Circulerende schildklierhormonen (TH) binden aan transport eiwitten (thyroglobuline bindend eiwit (TBG), thyroxine-bindend albumine (TBA) en albumine), terwijl een kleine fractie ongebonden is (free T4 (fT4) en free T3 (fT3)). De circulerende schildklierhormonen (TH) hebben vervolgens invloed op alle weefsels via TH receptoren, en ook een remmende werking op verdere TRH en TSH secretie door negatieve feedback, om een euthyroïde staat te behouden (TH en TSH binnen normaal waarden). Als de regulatie niet goed werkt, kunnen vormen van hyperthyreoïdie (overschot aan TH) of hypothyreoïdie (tekort aan TH) voorkomen, met alle gevolgen van dien.

Regulatie van schildklierhormonen in de weefsels

Schildklierhormonen kunnen door cellen worden opgenomen via transport eiwitten. Hierna worden ze geactiveerd (van T4 naar T3, of gedeactiveerd van T4 naar reverse T3 (rT3)) door deiodenase enzymen (in o.a. de lever, nier, skelet spieren en de schildklier) om het transcriptie proces in de cel kern te beïnvloeden (7).

Pathofysiologie

De invloed van schildklier hormoon is het duidelijkst zichtbaar in de complexe, ernstige en mogelijk levensbedreigende symptomen geassocieerd met hypo- en hyperthyreoïdie (tekort en overschot aan circulerende schildklierhormonen, respectievelijk) – die kunnen variëren van gewichtstoename, droge huid, haar verlies, moeheid, dyslipidemie, stemmingsproblemen en zelfs coma in het geval van

hypothyreoïdie (5,6), en gewichtsverlies, stemmingsproblematiek, atriumfibrillatie, spierverval en osteoporose in het geval van hyperthyreoïdie (5,8).

Schildklier en veroudering

Gezien de belangrijke rol die het schildklierhormoon speelt in meerdere fysiologische processen, is het niet verassend dat het ook van invloed is op veroudering en levensduur. Veroudering wordt beschreven als een proces dat wordt gedreven door een aantal kenmerkende processen (de zogenaamde hallmarks): primair (genoom instabiliteit, telomeer verkorting, epigenetica, verlies van proteostase), antagonistisch (ontregelde nutrient-sensing, mitochondriële dysfunctie, cellulaire senescentie) en integratief (stam cel depletie, veranderde intercellulaire communicatie) (4). Meerdere van deze processen zouden door schildklier hormoon beïnvloed kunnen worden en meerdere studies hebben aangetoond dat er een relatie bestaat tussen schildklier status en veroudering (9-11).

In proefdieren is een lager niveau van schildklierhormonen geassocieerd met een langere levensduur (10,12,13). Dit zou kunnen komen doordat lagere TH niveaus aan een lagere basale stofwisseling zouden kunnen bijdragen en dus aan een lagere productie van schadelijke reactieve zuurstof radicalen (ROS), die normaliter tot meer cel senescentie zouden kunnen leiden (14).

In mensen zijn lagere spiegels van schildklierhormonen, binnen normaalwaarden, geassocieerd met gezonde veroudering (11,17). Ook is de prevalentie van hoog normaal TSH hoger in oudere populaties (18). Daarnaast heeft een gerandomiseerde studie aangetoond dat het behandelen van subklinische hypothyreoïdie (verhoogd TSH met normale TH spiegels) in mensen van 65 plus geen duidelijke voordelen heeft (19) en hebben andere studies aangetoond dat levensduur in families geassocieerd is met (een familiale aanleg voor) hogere TSH waarden (19,20).

Dit proefschrift en hypothese

Dit proefschrift onderzoekt de schildklier-as in mensen uit de Leiden Langleven Studie (LLS). De LLS is in 2002 opgezet om de rol van verschillende genotypes en fenotypes in gezonde veroudering te onderzoeken door mensen te includeren uit langlevende families. Oorspronkelijk zijn mensen in Nederland geïncludeerd die voldeden aan de volgende criteria: vrouwen van 91 of ouder, en mannen van 89 jaar of ouder, die tenminste een broer of zus hadden doe ook voldeed aan dezelfde leeftijdscriteria. Vervolgens zijn de nakomelingen van deze proefpersonen geïncludeerd, met hun huidige partner als controle groep. In totaal zijn er 421 families geïncludeerd (22).

Het is eerder aangetoond dat nakomelingen een lagere mortaliteit en morbiditeit hebben dan controles (21). Daarnaast werd ook een associatie gevonden tussen familiale langlevendheid en lagere schildklier functie (11) en ook lagere T3 niveaus (17). Dit lijkt niet in alle cohorten van langlevende families het geval. In een andere populatie van langlevenden, Ashkenazi families en hun nakomelingen, was deze associatie tussen schildklier en langleven niet gevonden (20).

In 2015 zijn tijdens de Switchbox studie schildklier status hormonen in nakomelingen en controles van de LLS gemeten om de 10 minuten over een periode van 24 uur (23). Gedurende deze hele tijd hadden nakomelingen gemiddeld 0.8 mU/L hogere TSH spiegels dan controles, in afwezigheid van verschillen in schildklierhormoon spiegels (TH) tussen de twee groepen (23). Het is al uitgezocht dat de TSH bioactiviteit tussen deze twee groepen niet verschillend is (23).

Dit proefschrift richtte zich op het onderzoeken van het mechanisme achter het verschil in circulerend TSH tussen nakomelingen en controles uit de LLS. De hypothesen waren dat:

- 1) Nakomelingen van langlevende families een lagere respons van de schildklier hebben op TSH, en daardoor hogere TSH spiegels nodig hebben om voldoende TH te produceren

en/of

- 2) Nakomelingen van langlevende families een hogere afbraak van TH hebben dan controles, waardoor hogere TSH spiegels nodig zijn om adequate TH spiegels te onderhouden.

Deze hypothesen zijn onderzocht door middel van twee studies met schildklier geneesmiddelen (TSH en T3 studie) in nakomelingen en controles uit de LLS.

Opzet van studies

In **hoofdstuk 2** wordt de studieopzet toegelicht. De proefpersonen uit de LLS die in aanmerkingen kwamen voor de TSH en T3 studie waren deelnemers uit Switchbox waarvan de nakomelingen uit langlevende families een hoger TSH hadden dan controles. In de periode tussen november 2016 en december 2017 hebben 30 proefpersonen aan TSH studie deelgenomen en in de periode tussen augustus 2017 en september 2018 hebben 27 proefpersonen aan de T3 studie deelgenomen.

De TSH studie bestond uit vier aansluitende dagen in het LUMC, waar op de eerste studiedag een baseline (nul meting) bloedafname werd verricht, 0.1mg/ml recombinant humaan TSH (rhTSH) werd toegediend via intramusculaire injectie en vervolgens 16 bloedafnames zijn verricht (13 verdeeld over studiedag 1, en 1 op studiedag 2,3 en 4) om de kinetiek van schildklier hormonen te meten.

De T3 studie bestond uit vijf aansluitende dagen in het LUMC, waar op de eerste studiedag een baseline (nul meting) bloedafname verricht werd, 100mcg (4x25mcg) triiodothyronine (T3) ingenomen werd met water, en vervolgens 28 bloedafnames verricht werden (24 verdeeld over studiedag 1, en 1 op studiedag 2,3,4 en 5).

Bij beide studies is bloed afgenomen en verzameld in serum, EDTA plasma, vol bloed en PAX gene buizen, zijn ECG opnames gemaakt tijdens de keuring, waren de maaltijden op studiedag gestandaardiseerd en hebben alle proefpersonen meerdere vragenlijsten ingevuld o.a. over hun beweging, slaap en gewoontes.

Het primaire doel van deze studies was om de twee hypothesen betreffende de schildklier as in nakomelingen uit langlevende families en controles experimenteel te testen door middel van toediening van TSH en T3 hormoon.

Het secundaire doel van de studies was om (verschillen in) secundaire effecten van schildklier hormonen in nakomelingen en controles te onderzoeken, op weefsels en processen die door het schildklier as beïnvloed worden, zoals het bot weefsel en het immuunsysteem.

Resultaten pilot metingen rhTSH studie

De uitslagen van pilot metingen van rhTSH staan beschreven in **hoofdstuk 3**. De pilot studie bestond uit metingen van schildklier parameters (TSH, T4, fT4, T3, fT3 en Tg) in zes (n=6) deelnemers van de TSH studie op alle 17 tijdstippen van bloedafname. Het doel hiervan was om de optimale tijdstippen te identificeren voor metingen van schildklier parameters in gezonde ouderen na toediening van rhTSH, en dus de optimale meetpunten voor metingen in het hele cohort van de rhTSH studie (n=30). De resultaten tonen aan dat circulerend TSH hormoon de meeste variatie vertoont in de eerste 8 uur na rhTSH toediening, terwijl de concentratie van T4, fT4, T3 en fT3 begon te variëren vanaf 2u na rhTSH toediening, waarschijnlijk doordat eerst nieuwe aanmaak en secretie van deze hormonen gestimuleerd moest worden door het verhoogde TSH. Het Tg hormoon liet het minste variatie zien en ook pas later: 24, 48 en 72u na toediening van rhTSH.

Resultaten TSH studie

De resultaten uit de TSH studie worden in **hoofdstuk 4** beschreven. Om te onderzoeken of nakomelingen uit langlevende families een lagere respons van de

schilddklier hebben op TSH dan controles, zijn bij de TSH studie schilddklierhormonen gemeten in proefpersonen (15 nakomelingen en 15 controles) na intramusculaire toediening van 0.1mg rhTSH. Er zijn geen ernstige bijwerkingen waargenomen. Een persoon is uit de analyses geexcludeerd omdat verdacht werd dat het hier per abuis om intraveneuze i.p.v. intramusculaire rhTSH toediening ging. Bij de overige proefpersonen (n=29) stegen de TSH spiegels gedurende studiedag 1, en herstelden ze zich richting de waarde van de nul meting op studiedag 2, 3 en 4. De concentraties van circulerende schilddklier hormonen stegen ook, zoals verwacht na rhTSH toediening die de schilddklier stimuleert om deze aan te maken. De area under the curve (AUC) gedurende de studiedagen werd berekend per hormoon, als maatstaaf voor totale hormoon spiegels. De ratio van fT4 AUC en TSH AUC ($AUC\ fT4/AUC\ TSH$) was significant lager in nakomelingen dan in controles (de gemiddelde waarden waren respectievelijk 1.6 en 2.2). Dit betekent dat bij nakomelingen per hoeveelheid circulerend TSH minder circulerend fT4 werd geobserveerd tijdens de studie. De $AUC\ Tg/AUC\ TSH$ ratio was ook lager in nakomelingen dan in controles (de mediaan waarden waren 2.1 en 3.2, respectievelijk). Dezelfde trend werd met $AUC\ T3/AUC\ TSH$ ratio geobserveerd, echter was deze niet statistisch significant. Deze bevinding ondersteunt de eerste hypothese uit dit proefschrift, namelijk dat nakomelingen een lagere respons van de schilddklier hebben op TSH dan controles.

Resultaten T3 studie

In **hoofdstuk 5** staan de eerste resultaten genoemd van de T3 studie: de schilddklierhormoon en TSH spiegels na orale toediening van 100 microgram T3 hormoon. Om te onderzoeken of nakomelingen uit langlevende families een hogere afbraak van TH hebben dan controles, zijn bij de T3 studie de schilddklierhormonen gemeten in proefpersonen (14 nakomelingen en 13 controles) na T3 toediening. Deze studie gaf twee belangrijke bevindingen.

Ten eerste, na T3 toediening was de concentratie van circulerend T3 en fT3 in nakomelingen en controles vergelijkbaar. Ten tweede, circulerend TSH en de daling

(%) van TSH concentratie, als maat voor de negatieve feedback van T3 op TSH, was ook vergelijkbaar tussen nakomelingen en controles. Deze twee bevindingen steunen het verwerpen van de tweede hypothese, namelijk dat nakomelingen een hogere afbraak van TH hebben dan controles. De uitkomsten van de T3 studie tonen aan dat nakomelingen en controles na toediening van dezelfde dosering van T3, vergelijkbare spiegels hebben van T3 en fT3, en een vergelijkbare negatieve feedback hebben op TSH.

Een tekort van deze studie is dat de precieze afbraak van TH alleen meetbaar zou zijn als de studie zou zijn uitgevoerd met radioactief gemarkeerd T3, wat hier niet het geval was. Echter is in deze studie een zeer hoge meet frequentie gebruikt, waardoor toch met enige zekerheid conclusies getrokken kunnen worden over concentraties van schildklier-as hormonen in nakomelingen en controles.

Bot markers in TSH studie

In **hoofdstuk 6** staan de eerste secundaire uitkomsten van de TSH studie beschreven: de botmarkers in TSH studie. Het bot is een dynamisch weefsel dat continu wordt afgebroken en opgebouwd, wat in het bloedsomloop gemeten kan worden door bepalingen van circulerend CTX (botaafbraak marker) en P1NP (botaanmaak marker). Het effect van TSH op bot staat in de wetenschappelijke literatuur ter discussie. Bij de nul meting (zonder TSH toediening) van botmarkers hebben wij gevonden dat nakomelingen (n=14) lagere botmarkers hadden dan controles (n=15). Dit verschil werd zowel bij mannen als bij vrouwen waargenomen: zowel mannelijke als vrouwelijke nakomelingen hadden lagere botmarkers in vergelijking met mannelijke en vrouwelijke controles respectievelijk. Na rhTSH toediening stegen de botmarkers in beide groepen op vergelijkbare wijze. Dit kan het resultaat zijn geweest van TSH of van de mede stijgende TH, daar kan in gezonde proefpersonen geen onderscheid tussen gemaakt worden omdat deze parameters nauw met elkaar verbonden zijn.

Conclusie en toekomst perspectieven

Met de steeds groter wordende populatie van ouderen, en de stijgende prevalentie van chronische ziekte en de gezondheidszorg kosten die daarmee gepaard gaan, wordt steeds meer aandacht besteed aan het onderzoek van de processen achter gezond oud worden en gezond lang leven.

De schildklier speelt een essentiële rol in complexe fysiologische processen en wordt veel geïmpliceerd in processen die veroudering drijven, wat het een uitstekende kandidaat maakt voor onderzoek en innovatie op dit gebied.

Dit proefschrift beschrijft voor het eerst de concentraties van schildklierhormonen in een gezonde, oudere populatie na toediening van rhTSH als van T3 – met als subgroepen de nakomelingen uit langlevende families en de controles uit Leiden Langleven Studie.

Het is eerder in de Switchbox studie aangetoond dat nakomelingen uit lang levende families een andere schildklier as huishouding hebben dan controles, namelijk hogere TSH spiegels zonder verschillen in TH spiegels, terwijl deze parameters direct aan elkaar verbonden zijn middels feedback en feedforward regulatie. Dit proefschrift onderzocht het mechanisme achter dit verschil door twee interventie studies: een met TSH hormoon en een met T3 hormoon toediening, in deze nakomelingen en controles.

Na toediening van rhTSH, hebben nakomelingen uit langlevende families een lagere respons van de schildklier dan controles. Deze bevinding impliceert dat het hoogstwaarschijnlijk de schildklier respons op TSH is wat het oorspronkelijk gevonden verschil in TSH tussen nakomelingen en controles drijft. Nakomelingen lijken een lager schildklier respons op TSH te hebben, waardoor zij mogelijk een hogere concentratie TSH nodig hebben om voldoende TH te produceren. In vervolgonderzoek zou het interessant zijn om de rol van de TSH receptor als

mogelijke factor in de verschillen in schildklier respons tussen nakomelingen en controles te onderzoeken.

Na toediening van T3 hormoon, in de tweede studie, werden geen verschillen gevonden in de concentraties van schildklierhormonen en de negatieve feedback op TSH tussen nakomelingen uit langlevende families en controles. Op basis van deze bevindingen is de tweede hypothese van dit proefschrift minder waarschijnlijk – nakomelingen uit langlevende families lijken niet een hoger TSH en vergelijkbare TH als controles te hebben door een verschil in TH afbraak en feedback op TSH.

Concluderend, het is bekend dat nakomelingen uit lang levende families van de Leiden Langleven Studie lagere mortaliteit en morbiditeit hebben dan controles. Ook is bekend dat een subgroep hiervan in fysiologische staat hogere TSH spiegels hebben dan controles, in afwezigheid van verschillen in TH spiegels. Het mechanisme hierachter lijkt een lagere respons van de schildklier op TSH in nakomelingen dan in controles, wat in dit proefschrift voor het eerst is onderzocht en aangetoond.

Dit impliceert de mogelijke betrokkenheid van of hogere TSH spiegels, of bijvoorbeeld de TSH receptor, in langlevendheid. De TSH receptor is behalve op de schildklier ook aanwezig op andere weefsels, zoals bot en mogelijk ook cellen van het immuun systeem. Via dit mechanisme zou de schildklier invloed kunnen hebben op het menselijk lichaam en langlevendheid.

Gezond oud worden blijft een complex en genuanceerd onderwerp waarnaar veel verder onderzoek nodig is. Dit proefschrift bevestigt het belang en de implicatie van de schildklier as in langlevendheid en gezond oud worden, voornamelijk door een verschil in respons van de schildklier op TSH.

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Curriculum Vitae

Ana Žutinić was born on the 10th of June 1988 in Zagreb, Croatia. She attended primary education in Croatia and obtained the International General Certificate of Secondary Education and International Baccalaureate at the International School of The Hague in The Netherlands in 2006.

She commenced studies at Leiden University the following year, where she earned both Bachelor and Master degrees in Biomedical Sciences and Medicine, with an exchange to Heidelberg University and extracurricular laboratory work on diabetic nephropathy with the group of prof. dr. J.A. Bruijn. During this time she was active as a teaching assistant, Board member of Leiden Model United Nations Foundation (winning the Harvard World MUN Award), member of the Leiden Student Council, President of International Student Network, President of International Student Representation and member of the Van Bergen Fund Advisory Board. She is an alumna of the Leiden Leadership Program of Leiden University Honors Academy.

In 2016, she started a PhD project as co-principal investigator and lead trial physician where she set up and managed two EU Horizon 2020 studies with drugs investigating healthy aging in participants from Leiden Longevity Study, which she completed in two years. These studies were part of an EU Horizon 2020 consortium THYRAGE (2016-2021) investigating the role of the thyroid axis in healthy aging and age related diseases. She defended her thesis in Leiden following the COVID-19 lockdown.

