# Thyroid axis challenges in Leiden Longevity Study

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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 intra-muscular (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 thyroidal 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 thyroidal 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 SPPS 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.

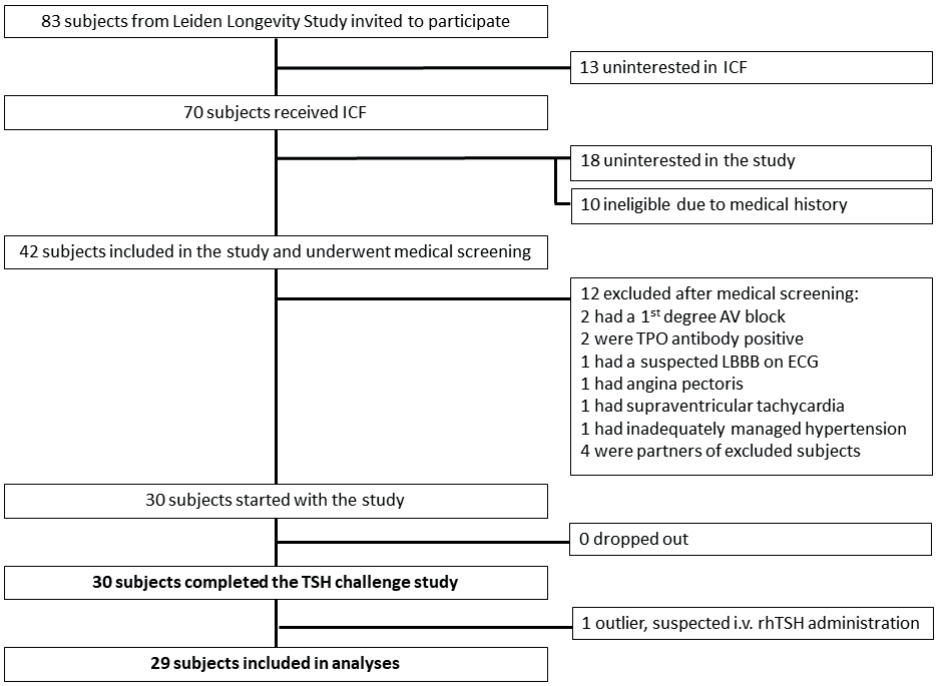


**Figure 1.** Recruitment and inclusion flowchart of subjects for the TSH challenge study. ICF: informed consent form, AV: atrioventricular, TPO: thyroid peroxidase, LBBB : left bundle branch block, ECG: electrocardiogram, i.v.: intravenous.

### *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 *years** | 93 (91–97) | 75 (69–85) |
| Age father *years** | 93 (73–96) | 78 (61–82) |
| Male *n (%)* | 8 (57) | 6 (40) |
| Age *years* | 69 (5) | 69 (6) |
| **ANTHROPOMETRICS** | | |
| BMI $kg/m^2$ | 25.8 (4.3) | 26.3 (4.4) |
| Weight *kg* | 78.1 (15.4) | 78.1 (15.0) |
| Height *cm* | 173.7 (10.9) | 171.9 (9.0) |
| Fat mass *kg* | 23.9 (7.2) | 26.7 (8.4) |
| Lean mass *kg* | 54.1 (12.9) | 50.7 (13.2) |
| **LABORATORY** | | |
| **MEASUREMENTS** | 71.2 (13.9) | 80.3 (8.4) |
| GFR $ml/min\ per\ 1.73m^2$ | 22.3 (4.1) | 24.6 (7.1) |
| AST *U/L* | 19.6 (5.3) | 22.1 (8.3) |
| ALT *U/L* | 3.3 (1.7) | 2.2 (1.0) |
| Baseline TSH *mU/L* | 13.9 (13.0-15.8) | 15.3 (14.3–15.7) |
| Baseline fT4* *pmol/L* | 4.6 (0.5) | 4.3 (0.5) |
| Baseline fT3 *pmol/L* | 10.7 (6.9–22.9) | 14.3 (10.2–33.6) |
| Baseline Tg* *μg/L* | | |

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) μg/L in F2-LLS and 50.5 (30.2-110.7) μg/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) μg/L and 2977 (1804-6512) μg/L, respectively, p=0.18.

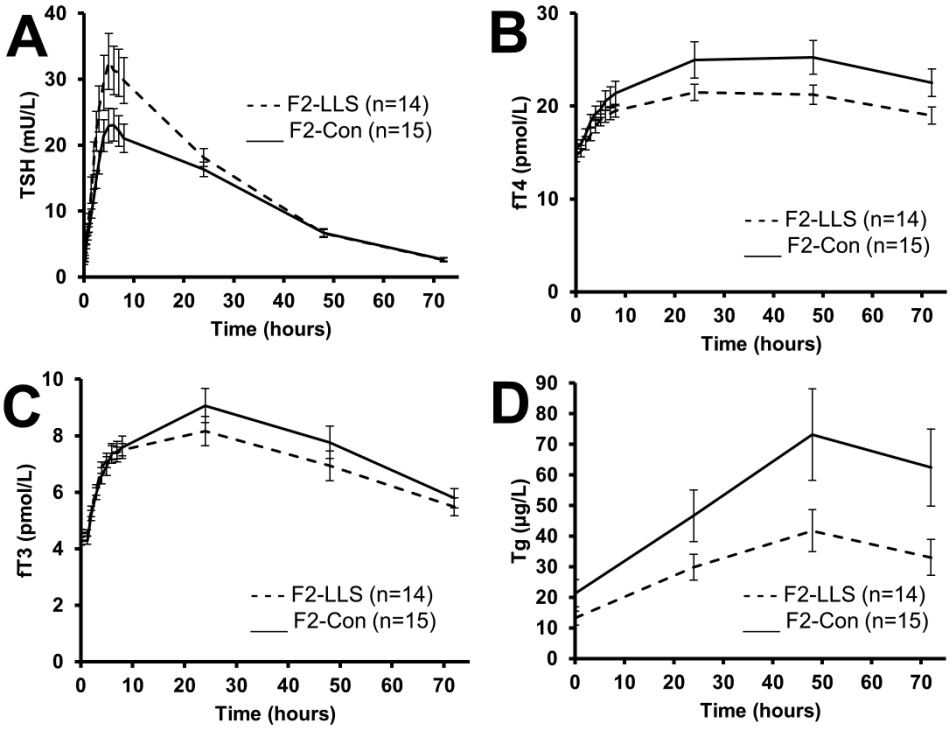


**Figure 2.** Four-day (72 h) profile of mean circulating thyroid stimulating hormone (TSH) and mean circulating thyroid hormones in members of long-lived families, F2-LLS (n=14) and their partners, F2-Con (n=15) following injection with 0.1mg recombinant human TSH. A) Mean circulating TSH, general linear 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.

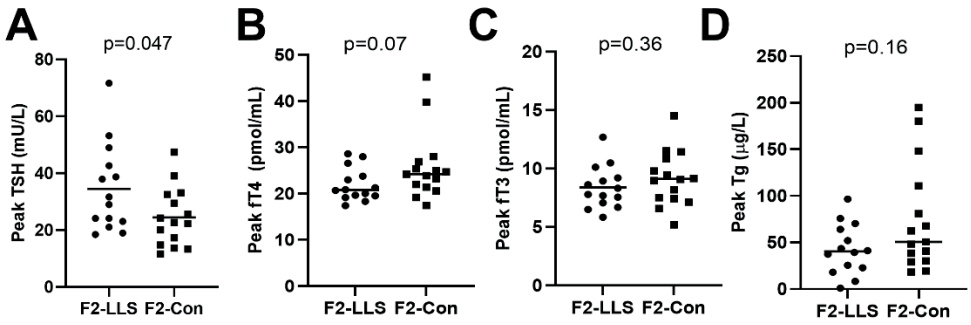


**Figure 3.** Peak values of circulating thyroid stimulating hormone (TSH) and circulating thyroid hormones in members of long lived-families, F2-LLS (n=14) and their partners, F2-Con (n=15) following injection with 0.1mg rhTSH. A) Peak values of TSH, horizontal line represents the mean, B) peak values 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) μg/mU and 3.2 (2.7-7.4) μg/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) μg/mU and 4.8 (3.3-6.2) μg/mU, respectively, p=0.04).

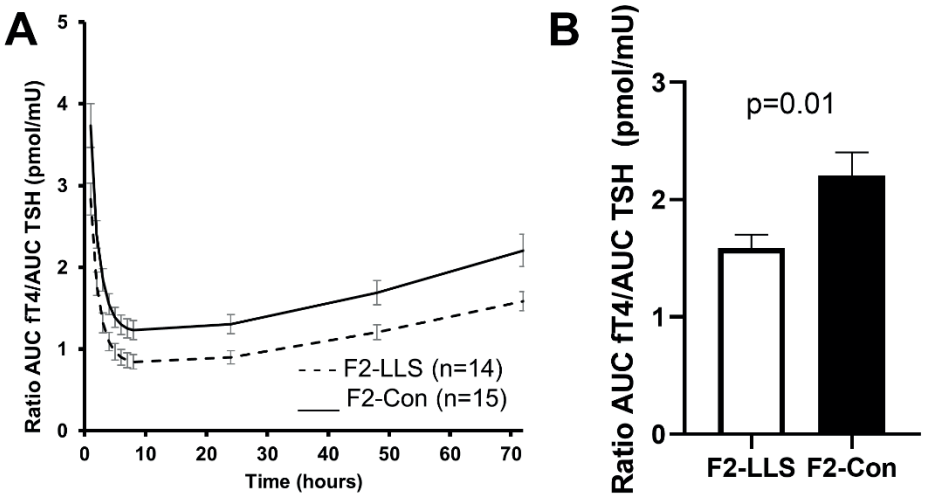


**Figure 4.** The ratio of circulating free T4 (fT4) to circulating thyroid stimulating hormone (TSH) in members of long-lived families, F2-LLS (n=14) and their partners, F2-Con (n=15), based on area under the curve calculations (AUC), following injection with 0.1mg recombinant human TSH. A) Four-day 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.

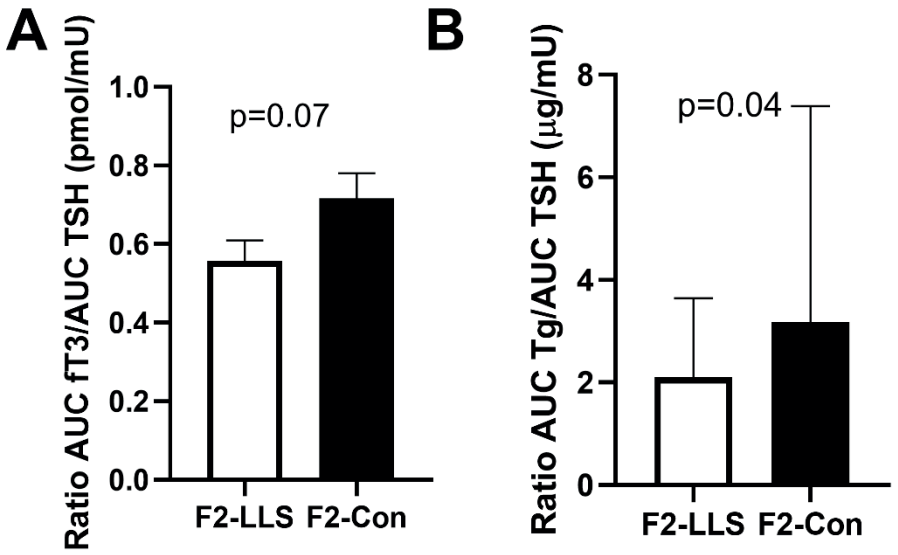


**Figure 5.** The ratio of circulating thyroid hormones free T3 (fT3) and thyroglobulin (Tg) to circulating thyroid stimulating hormone (TSH) in members of long-lived families, F2-LLS (n=14) and their partners, F2-Con (n=15), based on whole study (72 h) area under the curve calculations (AUC), following 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 de 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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