The endocrinology of familial longevity : time series analyses of different hormonal axes and their interrelationships

Spoel, E. van der

THE ENDOCRINOLOGY OF FAMILIAL LONGEVITY
TIME SERIES ANALYSES OF DIFFERENT HORMONAL AXES AND THEIR INTERRELATIONSHIPS
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THE ENDOCRINOLOGY OF FAMILIAL LONGEVITY

TIME SERIES ANALYSES OF DIFFERENT HORMONAL AXES

AND THEIR INTERRELATIONSHIPS

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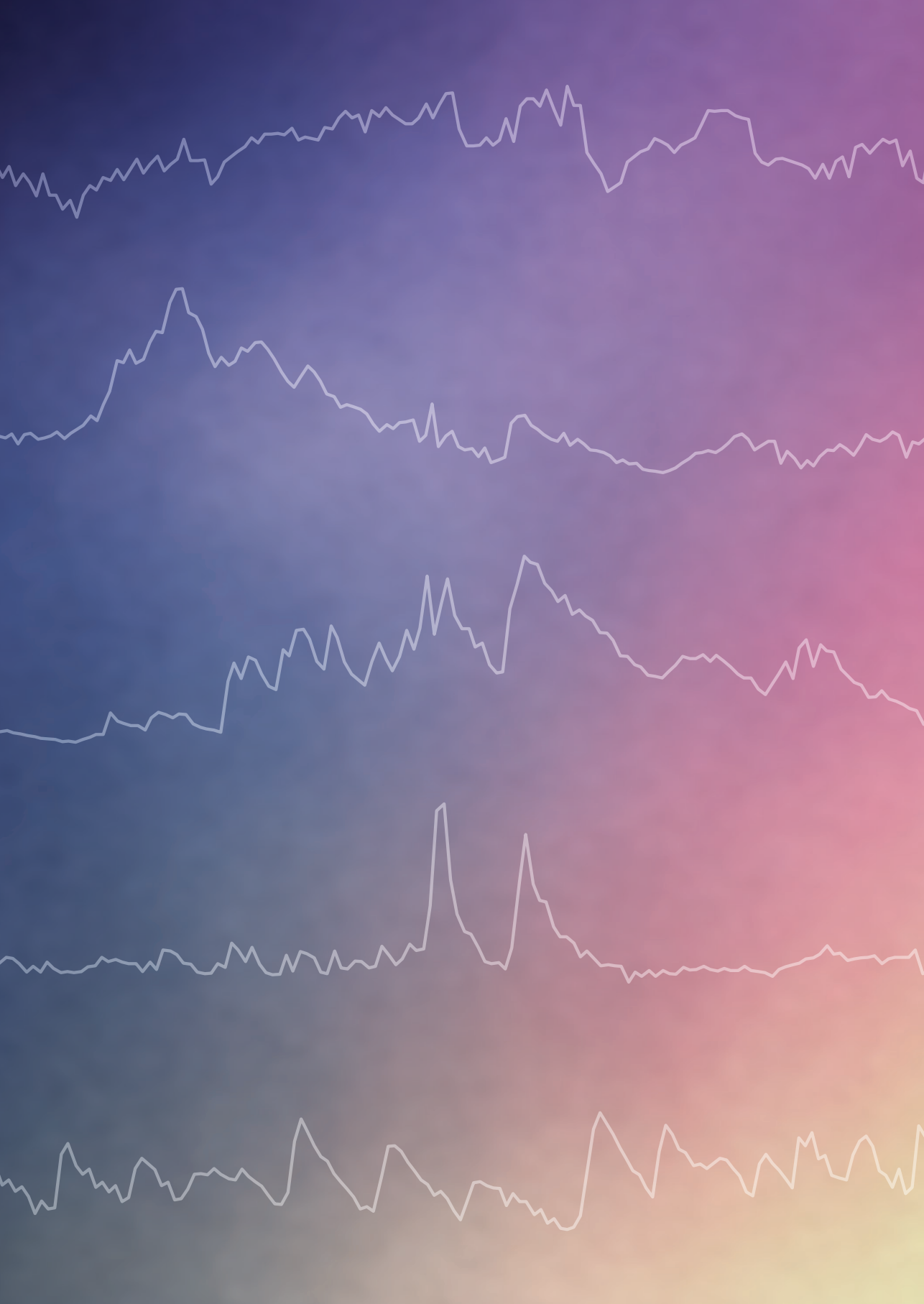
TABLE OF CONTENTS



1

General introduction and outline of this thesis

BACKGROUND

Studies in animal models indicate that altered central hormone signalling is associated with delayed ageing and longevity [1]. For example in the roundworm, insulin is secreted from neurosecretory cells in response to food cues, and single mutations in the insulin/insulin-like growth factor 1 (IGF-1) signalling pathway can double lifespan [2]. Also in mammals, mutations in the evolutionarily conserved growth hormone (GH)/IGF-1 pathway are associated with increased lifespan. Ames dwarf mice, which have a combined GH, prolactin, and thyroid-stimulating hormone (TSH) deficiency, live approximately 50% longer than wildtype controls [3]. Healthy ageing and longevity in humans are challenging to investigate, because of the relatively long lifespans and the difficulty to determine causality. Furthermore, proper controls are lacking in old age. Older persons are often compared to younger persons, but it is unclear whether differences thus identified are caused by differences between birth cohorts, selective survival or whether these reflect age-induced changes. To circumvent some of these methodological concerns the Leiden Longevity Study (LLS) was designed, in which 421 long-living families were included [4]. Along with nonagenarian siblings, their offspring, who have the propensity to reach old age in good health, together with their partners, as an environmental and age-matched control group, were included (see Figure 1 for the study design). Among the key findings from the LLS were the observations that the offspring had lower prevalence of myocardial infarctions, diabetes mellitus, hypertension, and metabolic syndrome compared to their partners [5, 6]. We also observed several differences in glucose and lipid metabolism as well as in endocrine features. Specifically, it was found that total secretion of TSH was higher in the offspring compared to their partners, but that there were no differences in the circulating thyroid hormone levels free triiodothyronine (fT3) and free thyroxine (fT4), nor in metabolic rate [7]. Familial longevity was found to be associated with a strong TSH-fT3 relationship, but not with major differences in hypothalamic-pituitary-adrenal (HPA) axis activity [8, 9].

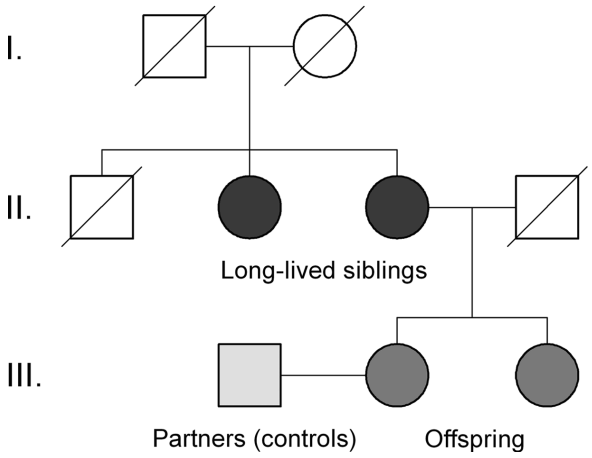


Figure 1. Study design of the Leiden Longevity Study.
Long-lived families with at least two Caucasian siblings fulfilling the age criteria (men ≥ 89 years and women ≥ 91 years) were included in the LLS, together with their offspring and partners of the offspring. Adapted from *PE Slagboom et al. Phil Trans R Soc Lond B Biol Sci. 2011 Jan 12;366(1561):35-42*.

GENERAL HYPOTHESIS

Maintenance and repair is of key importance for the proper functioning of cells, tissues, and integrated physiology. We hypothesize that the balance between investments in growth, development, and reproduction versus maintenance and repair is regulated by the brain. Specifically (the interplay of) hormones of the different hypothalamic-pituitary-target gland axes seem to be key regulators in constantly adjusting this balance to its optimal state. The optimal balance between these processes will be different for the different phases of the life cycle. Due to the accumulation of damage over time, requirements for maintenance and repair are hypothesized to increase with age. Furthermore, we hypothesized that longevity is associated with a prolonged ability to preserve an optimal balance throughout the different phases of life.

STUDY DESIGN

This PhD project was embedded into two International Consortia, Switchbox and Thyrage, funded by the European Union [10, 11]. In Switchbox, various physiological data and biomaterials have been collected over 24 h in 20 offspring and 18 partners from the LLS. Because pituitary hormones are secreted in a pulsatile manner and some exhibit a circadian rhythm, these hormones were measured in blood that was withdrawn every 10 min during 24 h to obtain reliable and informative data on pituitary hormone secretion. Concentrations of adrenocorticotropic hormone (ACTH) and cortisol from the HPA axis,

and of TSH had been measured before the start of this PhD project. Concentrations of luteinizing hormone (LH) and testosterone from the hypothalamic-pituitary-gonadal (HPG) axis, and of GH were measured during this PhD project. LH and testosterone concentrations were measured in blood withdrawn from 20 men only, of which 10 offspring and 10 partners. In the H2020 project Thyrage, one of the aims is to associate biomarkers of tissue maintenance with parameters of the thyroid axis in offspring and partners from the LLS. For this, an overview of possible and reliable biomarkers of tissue maintenance was written during this PhD project. For some of these biomarkers of tissue maintenance, especially bone turnover markers, it is known that they fluctuate over time. Before it is possible to associate biomarkers of bone turnover with parameters of the thyroid axis (and other pituitary hormones), we first need to determine the 24-h profile of bone turnover markers. To this end, bone turnover markers were measured in blood sampled every 4 h over 24 h during this PhD project.

OBJECTIVES

In this PhD project, I aimed to answer the following research questions:

1. Is familial longevity associated with altered endocrine features in the hypothalamic-pituitary-somatotropic axis?
2. Is familial longevity associated with altered endocrine features in the hypothalamic-pituitary-gonadal axis?
3. What are the interrelationships between hormones of the hypothalamic-pituitary-target gland axes in healthy older subjects?
4. What are the 24-h profiles of bone turnover markers in healthy older men and women?

METHODOLOGY

To examine these research questions, time series data on various hormone concentrations were collected. To analyse this type of data, specific methods for time series analysis are needed. Which method to use depends on the type of data and the research question. Below, the four time series analysis methods used in this PhD project are explained.

Cosinor analysis

To determine whether endocrine parameters display a sinusoidal circadian rhythm, cosinor analyses were performed. Cosinor analysis is a model-dependent method which fits a cosinor model to the raw data (see Figure 2 for an example). First, the rhythm detection test, also called the zero-amplitude test, was performed to test the overall

significance of the cosinor model. One of the circadian parameters calculated by the cosinor analysis is the midline estimating statistic of rhythm (MESOR), which is a circadian rhythm-adjusted mean based on the parameters of a cosine function fitted to the raw data. In addition, the amplitude is provided, which is the difference between the maximum and MESOR of the fitted curve. The acrophase represents the phase of the maximal value assumed by the curve [12].

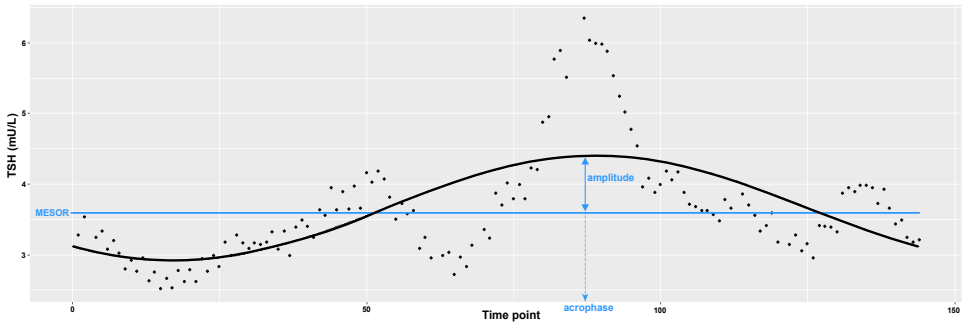


Figure 2. TSH concentration profile over 24 h of one participant with cosinor analysis.
The fit of the cosinor model is significant, indicating that this TSH concentration profile exhibits a circadian rhythm. The MESOR is indicated by the horizontal line, the amplitude by the solid arrow, and the acrophase by the dotted arrow.

Deconvolution analysis

By deconvolution analysis [13], a 24-h hormone concentration profile is decomposed into underlying secretory bursts, basal secretion, elimination of previously secreted hormone and random experimental variability using the Matlab software program. The algorithm first detrends the data and normalizes concentrations to numbers within the interval 0 to 1. Thereafter, successive potential pulse-time sets, each containing one fewer burst, were created by a smoothing process. Finally, a maximum-likelihood expectation deconvolution method estimated all secretion and elimination rates simultaneously for each candidate pulse-time set. Outcome parameters of main interest are basal (non-pulsatile) secretion, pulsatile secretion, the sum of basal and pulsatile secretion (total secretion), number of pulses per 24 h (secretory-burst frequency), interpulse regularity (Weibull gamma), mean pulse mass, and (fast and) slow half-life. Figure 3 presents an example of a GH concentration profile with indicated deconvolution parameters.

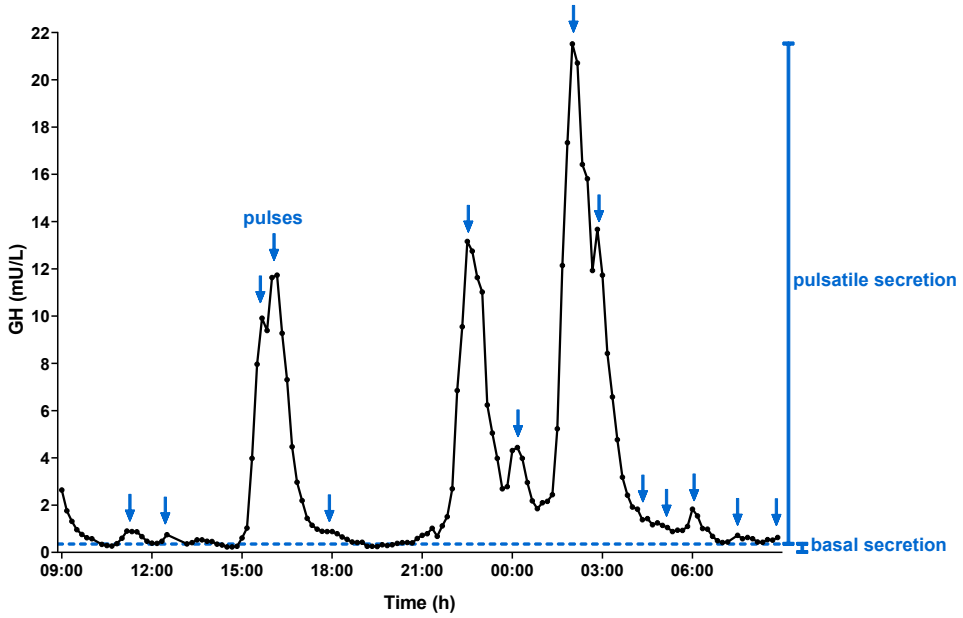


Figure 3. GH concentration profile over 24 h of one participant with deconvolution analysis parameters.

Pulses are indicated by the arrows, basal secretion by the dotted line and pulsatile secretion by the vertical line.

(Cross) Approximate Entropy

Approximate entropy (ApEn) is a measure for the strength of feedforward and feedback control signals in a hormone system. It is a scale- and model-independent statistic that quantifies the regularity of consecutive time-series data using the Matlab software program [14]. ApEn has high sensitivity and specificity (both > 90%) for analysis of hormone concentration measurements over 24 h. Low ApEn values imply that the sequence of time-series data is regular and that it contains many repetitive patterns, such as a sinus wave. High ApEn values indicate greater irregularity and randomness. Figure 4 presents the GH concentration profiles of two participants, one with a low ApEn value and one with a high ApEn. In neuro-endocrine time-series of a length of 50-300 data points, m (window length) = 1 is preferred, and for lengths $N \geq 60$, r (criterion of similarity) should be set to the predetermined value of 20% of the standard deviation (SD) of the individual subject time series [15]. Subsequently, the Jack-knifed ApEn (JkApEn) was calculated, which is a rigorous and objective cross-validation test that gives less bias in smaller samples than regular ApEn and it is more applicable for hormone data [16].

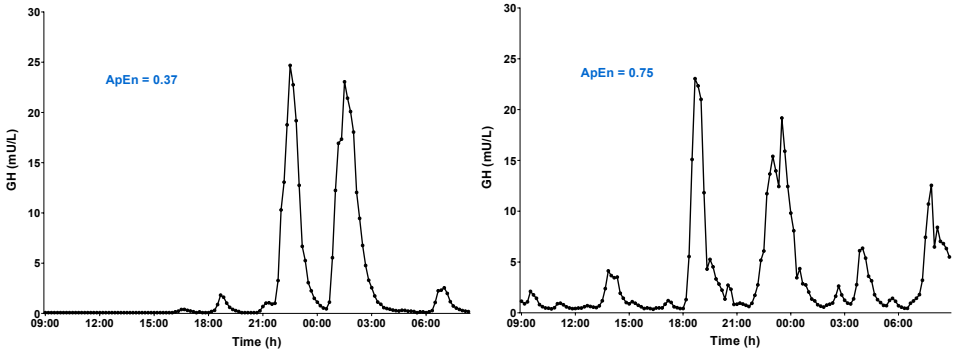


Figure 4. GH concentration profiles over 24 h of a participant (top) with a low ApEn, indicating a regular pattern, and a participant (bottom) with a high ApEn, indicating greater irregularity.

Bivariate cross approximate entropy (Cross-ApEn) quantifies joint pattern synchrony between two simultaneously measured time series, with lower cross-ApEn values signifying greater synchrony [17, 18]. Synchrony refers to pattern similarity, so to what extent sub patterns of window length *m* in time series A appear in time series B with a criterion of similarity *r*. Changes in the cross-ApEn reflect feedback and/or feedforward alterations within an interlinked axis [18].

Cross-correlation

Cross-correlation assesses the relative strength between two simultaneously measured hormonal time series for all possible time shifts by calculating linear Pearson's correlation coefficients [19, 20]. Hormone concentrations in time series A are compared pairwise with those of series B measured simultaneously (zero lag) or measured earlier or later (with a time lag). The unit of one lag time is the interval between two sampling points, so a lag time of 1 means that there is a delay of 10 min between two time series. Figure 5 presents a visual explanation of the cross-correlation procedure.

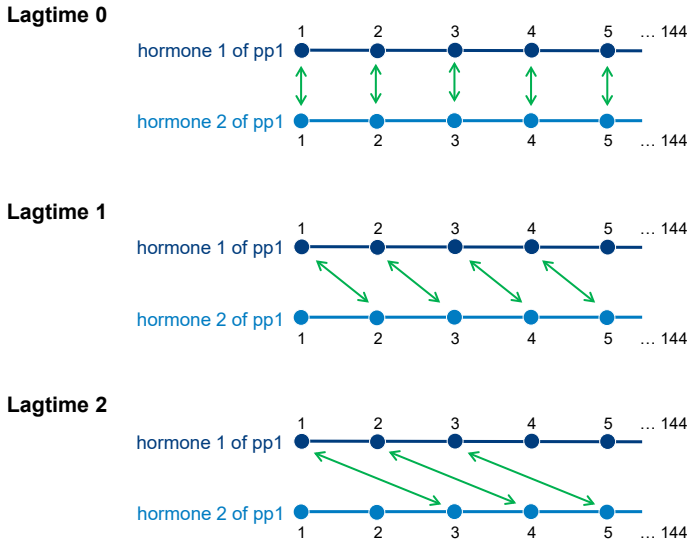


Figure 5. Explanation of cross-correlation.

The cross-correlation at lag time 0 is obtained when concentrations of hormone 1 are correlated with those of hormone 2 measured simultaneously in the same participant. The cross-correlations at lag time 1 and 2 are obtained when concentrations of hormone 1 are correlated with those of hormone 2 measured with a time lag of 10 and 20 min, respectively.

OUTLINE OF THIS THESIS

In **Chapter 2**, the question is addressed whether circulating IGF-1 axis parameters associate with old age survival and functional status in nonagenarians from the LLS. In **Chapter 3**, we use GH concentrations measured every 10 min over 24 h to derive and compare GH secretion parameters between offspring of long-lived families and their partners. In **Chapter 4**, we investigate the association between HPG axis parameters and familial longevity. In **Chapter 5**, we use 24-h time series data of pituitary hormones to investigate how changes in the different hormonal axes are correlated with each other over time. In **Chapter 6**, we determine the circadian rhythm of bone turnover markers in healthy older subjects.

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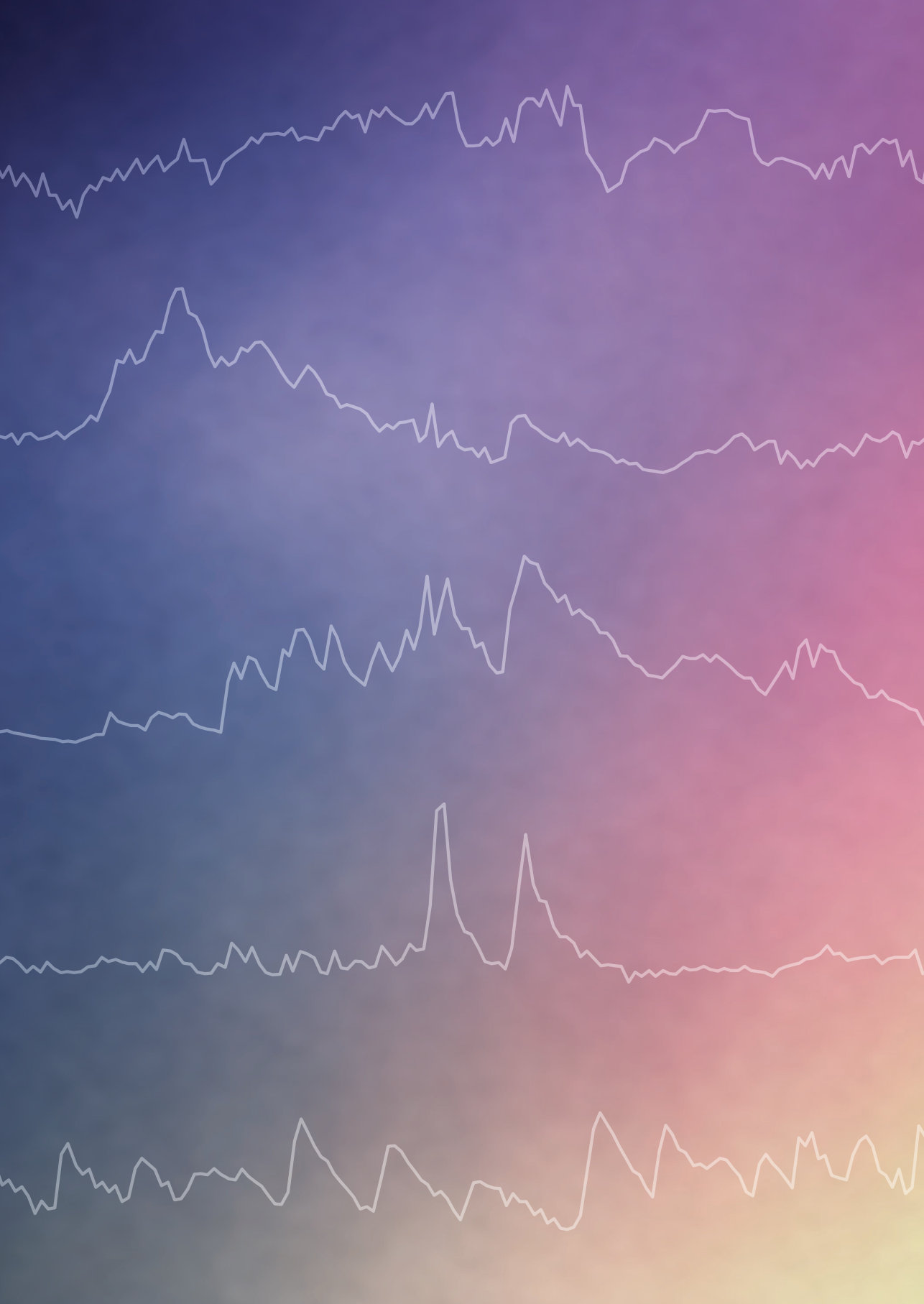

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Association analysis of insulin-like growth factor-1 axis parameters with survival and functional status in nonagenarians of the Leiden Longevity Study

Aging (Albany NY). 2015 Nov;7(11):956-63. doi: 10.18632/aging.100841

Evie van der Spoel, Maarten P. Rozing*, Jeanine J. Houwing-Duistermaat, P. Eline Slagboom, Marian Beekman, Anton J.M. de Craen, Rudi G.J. Westendorp, and Diana van Heemst, on behalf of the Leiden Longevity Study Group.*

ABSTRACT

Reduced insulin/insulin-like growth factor 1 (IGF-1) signalling has been associated with longevity in various model organisms. However, the role of insulin/IGF-1 signalling in human survival remains controversial. The aim of this study was to test whether circulating IGF-1 axis parameters associate with old age survival and functional status in nonagenarians from the Leiden Longevity Study. This study examined 858 Dutch nonagenarian (males ≥ 89 years; females ≥ 91 years) siblings from 409 families, without selection on health or demographic characteristics. Nonagenarians were divided over sex-specific strata according to their levels of IGF-1, IGF binding protein 3 and IGF-1/IGFBP3 molar ratio. We found that lower IGF-1/IGFBP3 ratios were associated with improved survival: nonagenarians in the quartile of the lowest ratio had a lower estimated hazard ratio (95% confidence interval) of 0.73 (0.59 – 0.91) compared to the quartile with the highest ratio (P_{trend} = 0.002). Functional status was assessed by (Instrumental) Activities of Daily Living ((I)ADL) scales. Compared to those in the quartile with the highest IGF-1/IGFBP3 ratio, nonagenarians in the lowest quartile had higher scores for ADL (P_{trend} = 0.001) and IADL (P_{trend} = 0.003). These findings suggest that IGF-1 axis parameters are associated with increased old age survival and better functional status in nonagenarians from the Leiden Longevity Study.

INTRODUCTION

The role of the evolutionarily conserved insulin/insulin-like growth factor (IGF-1) signalling (IIS) pathway in the regulation of lifespan is well documented in worms [1], flies [2], and rodents [3, 4]. Genetic mutations that inhibit IIS activation prolong lifespan in these organisms, particularly in the female sex. The involvement of IIS modulation in human longevity is less clear [5]. In agreement with the findings in model organisms, reduced IIS pathway activity was associated with old age survival in sporadic female octogenarians and different cohorts of nonagenarians [6, 7] as well as with better cognitive function [8]. Furthermore, centenarians were shown to be enriched for rare IGF-1R mutations associated with IGF-1 resistance [9]. As in mice, mutations causing growth hormone (GH) resistance resulting in low circulating levels of IGF-1 have been reported to confer protection against the development of cancer and diabetes in men [10].

Circulatory IGF-1 is mostly bound to any of six high affinity IGF binding proteins, of which IGF binding protein 3 (IGFBP3) is the most abundant. The IGFBP3 glycoprotein forms a complex with IGF-1 and an acid-labile component and serves as a reservoir of IGF-1 in the circulation. IGF-1 is only biologically active in its free form, which accounts for approximately 1% of total IGF-1. Therefore, the IGF-1/IGFBP3 molar ratio is considered a better indicator of IGF-1 bioavailability than total IGF-1 [11, 12]. Both IGF-1 and IGBP3 are under control of GH [13]. With age, levels of GH decline as do the levels of IGF-1 and IGFBP3 [14]. In contrast to the apparent beneficial effects associated with constitutively low GH/IGF-1 activity discussed above, lower serum IGF-1 levels in humans have also been associated with an increased risk of developing cardiovascular disease and diabetes [15].

In order to identify heritable determinants of longevity we set up the Leiden Longevity Study. This study includes nonagenarian siblings, recruited from 421 Caucasian families based on proband siblings that both exhibit exceptional longevity [16] and their offspring [17]. Using pathway analysis, a significant difference was detected between nonagenarians from the Leiden Longevity Study and a younger age group for the joint effect of genetic variation in the insulin/IGF-1 signalling pathway [18]. In seeming contrast, earlier we reported on the lack of differences in serum levels of IGF-1 axis parameter between middle-aged offspring of familial nonagenarians and controls [19]. However as not all offspring will inherit the favourable genetic predisposition for longevity of their long-lived parent it is unclear to what extent serum levels of IGF-1 axis parameter levels in middle-aged offspring are reflective of a constitutional phenotype predisposing to longevity. Therefore, in the current study we aim to examine whether circulating levels of

IGF-1, IGFBP3 and IGF-1/IGFBP3 molar ratio are associated with old age survival and functional status in nonagenarian siblings from the Leiden Longevity Study.

METHODS

In the Leiden Longevity Study, 421 families were recruited consisting of long-lived Caucasian siblings together with their offspring and the partners thereof. For the current study, data on IGF-1 and IGFBP3 levels were available for 858 of the 944 nonagenarian participants from the Leiden Longevity Study. After a median follow-up time of 3.4 years (range 0 – 11.5 years), 797 individuals (92.9%) had died. The Medical Ethical Committee of the Leiden University Medical Center approved the study and informed consent was obtained from all subjects. For details on enrolment please see previous publications [17, 19].

All serum measurements were performed with fully automated equipment. For IGF-1, IGFBP3, and insulin the Modular E170 was used, for glucose, high sensitivity C-reactive protein (hsCRP) and free triiodothyronine the Cobas Integra 800 was used, both from Roche, Almere, the Netherlands. The coefficients of variation of these measurements were all below 5%.

Global cognitive function was assessed with the Mini-Mental State Examination (MMSE) and functional status was assessed by Activities of Daily Living (ADL) and Instrumental Activities of Daily Living (IADL) scales as described previously [20]. ADL disability scores range from 0 points (fully dependent in all activities) to 20 points (fully independent in all activities). IADL disability scores range from 0 points (fully dependent in all activities) to 14 points (fully independent in all activities). MMSE scores range from zero points (very severe cognitive impairment) to 30 points (optimal cognitive function).

For each parent, we computed the sex and birth cohort cumulative hazards using the life tables of the Dutch population. Note that since both parents are deceased one minus the cumulative hazard equals the martingale residual. The martingale residual is defined as the difference between the event status (0 if alive, 1 if deceased) and the cumulative hazard at the observed age (current age or age at death). The sum of the martingale residuals measures the deviation of survival of the parents with respect to their birth cohort. Therefore, negative values indicate excess survival and positive values indicate excess mortality.

Nonagenarians were divided over four sex-specific strata according to their circulating levels of IGF-1, IGFBP3 or their IGF-1/IGFBP3 molar ratio. The association between quartiles of IGF-1 axis parameters and baseline characteristics of nonagenarians was assessed using a linear mixed model corrected for correlation of sibling data using robust standard errors clustered on family number. Distributions of continuous variables were examined for normality and logarithmically transformed when appropriate. Survival analyses were performed with a left truncated Cox proportional hazards model to correct for the delayed entry into the risk set according to age and the model was corrected for correlation of sibling data using robust standard errors clustered on family number. The Statistical Package STATA ("statistics and data") for Windows, version 12.0 SE, and the Statistical Package for the Social Sciences (SPSS) for Windows, version 20.0, were used for data analysis.

RESULTS

Baseline characteristics

The baseline features of the study population (*n* = 858) are displayed stratified for women (*n* = 528) and men (*n* = 330) in Table 1. The median age of women (93.6 years) was higher than that of men (91.4 years). Women and men also showed significant differences in circulating levels of IGFBP3 and the IGF-1/IGFPB3 molar ratio as well as in scores for cognition, functional status and circulating levels of free triiodothyronine (Table 1).

Table 1. Baseline characteristics of the study population

	Women	Men	*P* value
Participants (*N*)	528	330	
Age (years)	93.6 (92.2 – 95.3)	91.4 (90.1 – 93.7)	<0.001
Family mortality history score parents	-1.4 (-2.8 – -0.2)	-1.3 (-2.5 – -0.2)	0.94
IGF-1 [nmol/L]	10.1 (7.7 – 13.0)	10.1 (7.6 – 13.2)	0.98
IGFBP3 [mg/L] [†*]	3.2 (0.9)	2.7 (0.8)	<0.001
IGF-1/IGFBP3 molar ratio[*]	0.09 (0.08 – 0.11)	0.11 (0.10 – 0.13)	<0.001
Disability (points)			
Mini-Mental State Examination[~]	25 (21 - 28)	26 (24 – 28)	0.03
Activities of Daily Living (ADL)[#]	17 (13 – 19)	19 (17 – 20)	<0.001
Instrumental ADL[#]	7 (3 – 11)	10 (6 – 12)	<0.001
Non-fasted glucose [mmol/L][·]	6.1 (5.3 - 7.2)	6.1 (5.4 – 7.1)	0.81
Non-fasted insulin [mU/L]	21.0 (11.3 – 36.0)	23.0 (12.0 – 37.0)	0.39
Free triiodothyronine [pmol/L] [†]	4.0 (0.7)	4.1 (0.7)	0.04
High sensitivity C-reactive protein [mg/L][‡]	2.7 (1.3 – 5.4)	3.0 (1.4 – 6.6)	0.31

Unless specified otherwise, data are presented as median with interquartile ranges and analysed with a non-parametric Median Test. [†]data are presented as mean with standard deviation and analysed with linear regression. [*]data available for 527 women and 329 men; [~]data available for 467 women and 308 men; [#]data available for 488 women and 307 men; [·]data available for 527 women and 327 men; [‡]data available for 528 women and 329 men.

Association of IGF-1 axis parameters and survival

Nonagenarian siblings were divided over four sex-specific strata according to their circulating levels of IGF-1, IGFBP3 or their IGF-1/IGFBP3 molar ratio. In Table 2, we assessed the relation between quartiles of serum IGF-1 axis parameters and survival, using a left truncated Cox proportional hazards model to correct for the delayed entry into the risk set according to age. Table 2 shows that lower IGF-1/IGFBP3 molar ratios were associated with significantly lower hazard ratios (*P* for trend = 0.002). There was no interaction between sex-specific quartiles of IGF-1/IGFBP3 molar ratios and sex (*P* = 0.57). We found a proportional hazard ratio of 0.73 for nonagenarians in the quartile with the lowest IGF-1/IGFBP3 ratio, which is indicative of a 27% higher chance of survival compared

to nonagenarians in the quartile with the highest IGF-1/IGFBP3 ratio. Moreover, observed effects did not change during the course of follow-up (data not shown).

Table 2. Estimated hazard ratios for sex-specific quartiles of serum IGF-1 axis parameters

	Median (range) women	Median (range) men	Hazard ratio	*P* value
IGF-1 [nmol/L]				
Q1	6.3 (3.1 – 7.6)	6.5 (3.6 – 7.5)	0.89 (0.72 – 1.11)	0.30
Q2	8.7 (7.7 – 10.0)	9.0 (7.6 – 10.0)	0.81 (0.65 – 1.00)	0.05
Q3	11.4 (10.1 – 12.9)	11.4 (10.1 – 13.0)	0.94 (0.77 – 1.14)	0.52
Q4	15.8 (13.0 – 31.3)	15.3 (13.2 – 30.5)	1 (ref)	
		P for trend	0.15	
IGFBP3 [mg/L]				
Q1	2.2 (0.8 – 2.5)	1.8 (1.0 – 2.0)	1.11 (0.90 – 1.37)	0.35
Q2	2.8 (2.6 – 3.0)	2.3 (2.1 – 2.5)	1.04 (0.85 – 1.26)	0.72
Q3	3.3 (3.1 – 3.6)	2.8 (2.6 – 3.0)	0.99 (0.81 – 1.20)	0.89
Q4	4.2 (3.7 – 6.7)	3.5 (3.1 – 10.1)	1 (ref)	
		P for trend	0.31	
IGF-1/IGFBP3 molar ratio				
Q1	0.07 (0.05 – 0.08)	0.09 (0.02 – 0.10)	0.73 (0.59 – 0.91)	0.005
Q2	0.09 (0.08 – 0.09)	0.11 (0.10 – 0.11)	0.74 (0.59 – 0.92)	0.007
Q3	0.10 (0.09 – 0.11)	0.12 (0.11 – 0.13)	0.87 (0.72 – 1.06)	0.16
Q4	0.13 (0.11 – 0.21)	0.15 (0.13 – 0.29)	1 (ref)	
		P for trend	0.002	

Data are presented as estimated hazard ratios with 95% confidence intervals per sex-specific quartiles (Q) of IGF-1, IGFBP3 or IGF-1/IGFBP3 molar ratio as compared to highest quartile, analysed with cox regression adjusted for family relationship.

Association of IGF-1/IGFBP3 molar ratio and functional status

Next, we assessed the relation between quartiles of serum IGF-1 axis parameters and available baseline measures of functional status and health. Table 3 shows that lower IGF-1/IGFBP3 molar ratios in nonagenarians were associated with less physical disability. Compared to nonagenarians in the quartile with the highest IGF-1/IGFBP3 ratios, those in the quartile of the lowest IGF-1/IGFBP3 molar ratios had higher mean (95% CI) scores for

both Activities of Daily Living (ADL) (16.8 (16.3 – 17.4) vs 15.2 (14.5 – 15.9), *P* for trend = 0.001) and Instrumental Activities of Daily Living (IADL) (8.0 (7.4 – 8.7) vs 6.9 (6.3 – 7.5), *P* for trend = 0.003). The Mini-Mental State Examination (MMSE) did not significantly differ across quartiles. Interestingly, lower IGF-1/IGFBP3 molar ratios were associated with lower levels of non-fasted insulin.

Family mortality history score

Previously, we had calculated a family mortality history score describing the mortality of the parents of the nonagenarian siblings [21]. To assess whether a lower IGF-1/IGBP3 molar ratio is a feature of familial longevity, we compared the family mortality history score across quartiles of IGF-1/IGFBP3 molar ratio. Table 3 shows that lower IGF-1/IGFBP3 molar ratios in nonagenarian siblings were not associated with a lower family mortality history score (i.e. lower than expected mortality of the parents of the nonagenarian siblings).

Table 3. Baseline characteristics for quartiles of IGF-1/IGFBP3 molar ratio

	Q1	Q2	Q3	Q4	*P* for trend
Demographics					
Participants (*N*)	213	213	215	215	
Men (*N*, %)	82 (38.5)	82 (38.5)	83 (38.6)	82 (38.1)	0.95
Age (years)	93.5 (93.1 – 93.8)	93.3 (93.0 – 93.7)	93.4 (93.0 – 93.7)	93.1 (92.8 – 93.5)	0.27
Disability (points)					
Mini-Mental State Examination[~]	24.4 (23.7 – 25.2)	24.5 (23.7 – 25.2)	24.5 (23.8 – 25.1)	23.6 (22.8 – 24.4)	0.11
Activities of Daily Living (ADL)[#]	16.8 (16.3 – 17.4)	16.4 (15.7 – 17.1)	16.2 (15.6 – 16.8)	15.2 (14.5 – 15.9)	0.001
Instrumental ADL[#]	8.0 (7.4 – 8.7)	8.2 (7.6 – 8.8)	7.6 (7.0 – 8.1)	6.9 (6.3 – 7.5)	0.003
Serum parameters					
Non-fasted glucose [mmol/L][·]	6.2 (5.9 – 6.4)	6.4 (6.2 – 6.6)	6.4 (6.2 – 6.6)	6.4 (6.2 – 6.7)	0.10
Non-fasted insulin [mU/L]	16.3 (14.4 – 18.5)	20.7 (18.5 – 23.2)	20.9 (18.7 – 23.5)	23.3 (20.9 – 25.9)	<0.001
Free triiodothyronine [pmol/L]	4.0 (3.9 – 4.1)	4.1 (4.0 – 4.2)	4.1 (4.0 – 4.2)	4.1 (4.0 – 4.2)	0.23
High sensitivity C-reactive protein [mg/L][‡]	2.7 (2.3 – 3.2)	3.2 (2.7 – 3.8)	2.6 (2.2 – 3.1)	3.1 (2.6 – 3.7)	0.64
Family mortality history score parents	-1.7 (-1.9 – -1.4)	-1.8 (-2.1 – -1.6)	-1.5 (-1.8 – -1.3)	-1.5 (-1.8 – -1.3)	0.22

Unless specified otherwise, data are presented as mean with 95% confidence interval, analysed with linear regression adjusted for age and family relationship except for the variables sex, age and family mortality history score parents). [~]data available for 774 participants (197 in Q1, 193 in Q2, 189 in Q3, 195 in Q4); [#]data available for 793 participants (192 in Q1, 196 in Q2, 200 in Q3, 205 in Q4); [·]data available for 852 participants (212 in Q1, 212 in Q2, 213 in Q4); [‡]data available for 855 participants (212 in Q1).

DISCUSSION

This study aimed to explore the association of circulating IGF-1 axis serum parameters with survival and functional status in nonagenarian siblings from the Leiden Longevity Study. First we demonstrated that lower IGF-1/IGFBP3 molar ratios conferred a survival benefit at the age of ninety years or older. Secondly, lower IGF-1/IGFBP3 molar ratios were associated with better functional status at the age of ninety years or older.

Our outcomes support the recent observation that low IGF-1 levels predict survival in exceptionally long-lived humans [22]. Not only do we confirm that lower IGF-1 axis serum parameters are related with survival in the oldest old, but we also demonstrate that lower IGF-1 axis serum parameters are associated with better functional status. A large number of studies have reported an association between reduced IIS activity and longevity in various model organisms as well as in human studies showing life extending effects of reduced IGF-1 signalling [1-4, 7]. In these studies lifespan extending effects were mostly confined to females, unlike the results presented here. In contrast with our findings, Paolisso *et al.* observed higher serum IGF-1/IGFBP3 ratios in healthy centenarians when compared to aged controls [23]. Another study showed a higher prevalence of heterozygous mutations in the IGF-1R in Ashkenazi Jewish centenarians compared to controls with concomitant higher serum IGF-1 levels [9]. Moreover, they found a sex-specific increase in serum IGF-1 associated with a smaller stature in female offspring of centenarians, suggesting the involvement of reduced IGF-1R activity in human longevity [9]. Unfortunately, anthropometric data were not available in our current study. Therefore, we cannot draw firm conclusions as to how the IGF-1/IGFBP3 ratios correlate with the underlying IGF-1 signalling activity in our study population.

It has been suggested that low levels of IGF-1 and/or reduced IGF-1 bioavailability form part of a survival response that can be constitutively active in long-lived individuals as well as elicited by diverse forms of stress, including metabolic stress, genotoxic stress and inflammation [24]. In hospitalized elderly patients, frailty, impairments and mortality were associated with a distinct biomarker signature that comprised higher levels of inflammatory markers and lower levels of growth factors and anabolic hormones, including IGF-1 and free triiodothyronine [25]. In our study, levels of CRP and free triiodothyronine did not differ across quartiles of the IGF-1/IGFBP3 molar ratios, while functional status was better in the quartile with the lowest IGF-1/IGFBP3 ratio, in line with the observed survival benefit.

A limitation of our study is that information about medical history, medication use, and specific causes of death is lacking. Previous research suggested that enhancement of insulin sensitivity was a key mediator of the increased longevity of hypopituitary, GH-resistant, and calorie-restricted animals [26]. Moreover, preserved insulin sensitivity was also shown to be a key phenotype of human longevity, both at middle age [27] and at extremely high ages [28]. Previously, we observed lower fasted levels of glucose and insulin when the offspring of the included nonagenarians were compared to an age-matched control group [29]. It is a limitation of the current study that all blood parameters, including IGF-1, IGFBP3, glucose and insulin were determined in non-fasted samples collected at a random moment of the day and that data on insulin sensitivity and food intake were lacking. Circulatory levels of IGF-1 will decrease while levels of its binding proteins (notably IGFBP1) will increase in response to reductions in food intake, in particular the intake of protein [30]. Nevertheless, our finding that lower IGF-1/IGFBP3 molar ratios were associated with relatively lower circulating levels of insulin is suggestive of better insulin sensitivity and in accordance with other recent human data [31]. IGF-1 has structural and functional homology with insulin, and it has been suggested that insulin resistance might lead to increased IGF-1 bioavailability to compensate for reduced insulin action. Another limitation of our study is its cross-sectional design which precludes causal inference of the observed associations.

Because we found that lower IGF-1/IGFBP3 ratios were associated with better old age survival in nonagenarians of the LLS, we assessed whether nonagenarian IGF-1/IGFBP3 ratios were associated with a family mortality history score describing the mortality of the parents of the nonagenarian siblings. Our findings on lack of association between nonagenarian IGF-1/IGFBP3 ratios and family mortality history score are in accordance with the lack of difference in IGF-1 axis parameters, including IGF-1, IGFBP3 and the IGF-1/IGFBP3 molar ratios previously observed between middle-aged offspring of familial nonagenarians and controls. These disparate results suggest that the possible benefits of low IGF-1/IGFBP3 ratios may differ according to age and birth cohorts. One of the possible explanations is that with advancing age, the IGF-1/IGFBP3 ratio changes and that those individuals that adapt to a lower IGF-1/IGFBP3 ratio have a survival advantage in old age. Another possibility is selective survival of participants with constitutively lower IGF-1/IGFBP3 ratios. It is possible that selective advantage of variation in the IIS pathway may only become detectable at advanced ages. In line, the association between FOXO3A and longevity was for example found to be stronger in centenarians than in nonagenarians [32]. In our study, a considerable percentage of included nonagenarians (23.8%) also reached an age of 100 years or more. Amongst others, another possible explanation for

these contrasting observations could be differences in imprinting of IGF-1 axis genes, reflecting historical differences in maternal nutrition between generations [33].

In conclusion, we showed that in nonagenarian siblings IGF-1/IGFBP3 molar ratios are associated with better survival and functional status. These preliminary findings support the involvement of IGF-1 signalling in modulating human longevity.

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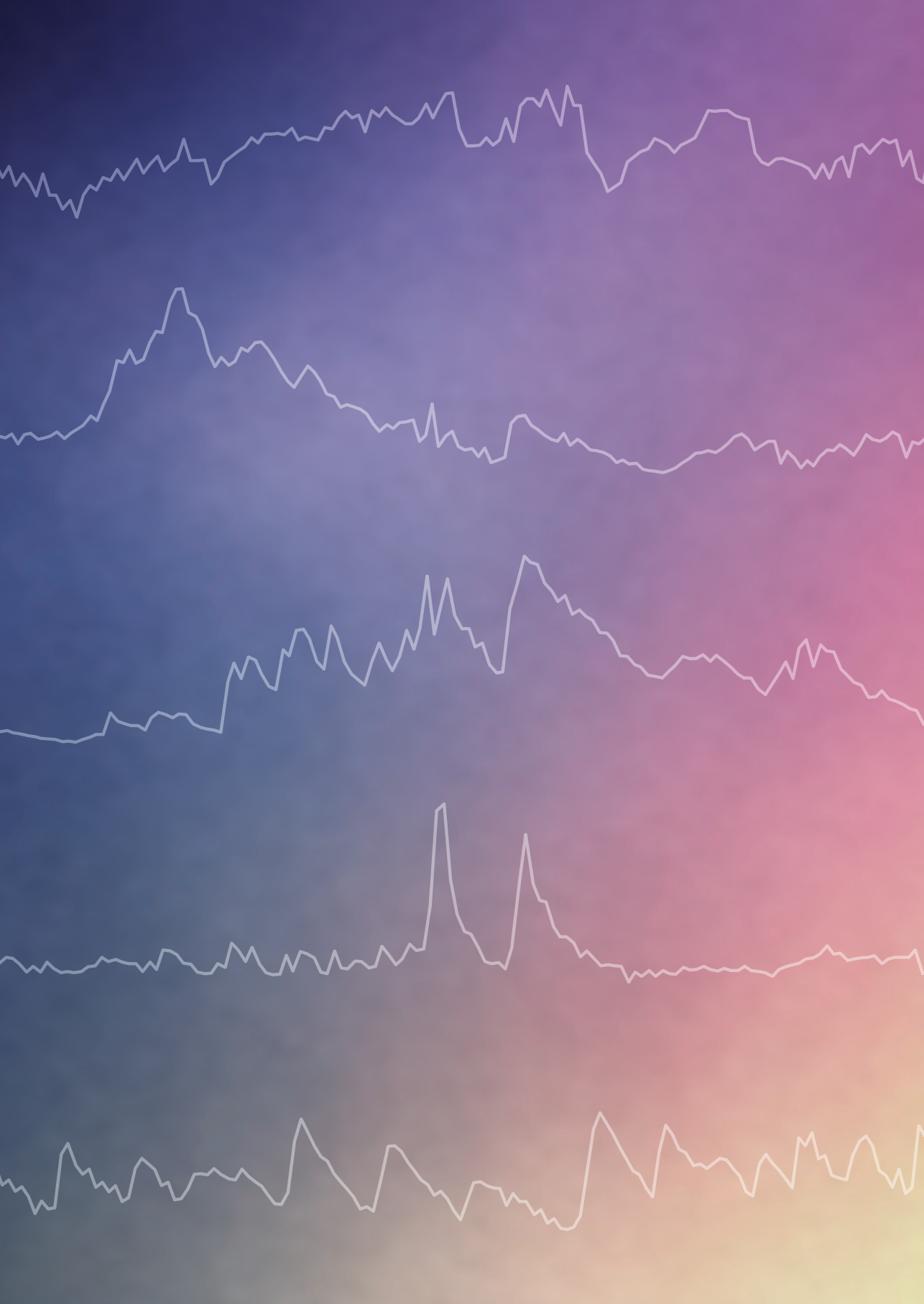

3

Growth hormone secretion is diminished and tightly controlled in humans enriched for familial longevity

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ABSTRACT

Reduced growth hormone (GH) signaling has been consistently associated with increased health and life span in various mouse models. Here, we assessed GH secretion and its control in relation with human familial longevity. We frequently sampled blood over 24 h in 19 middle-aged offspring of long-living families from the Leiden Longevity Study together with 18 of their partners as controls. Circulating GH concentrations were measured every 10 min and insulin-like growth factor 1 (IGF-1) and insulin-like growth factor binding protein 3 (IGFBP3) every 4 h. Using deconvolution analysis, we found that 24-h total GH secretion was 28% lower (P = 0.04) in offspring (172 (128–216) mU/L) compared with controls (238 (193–284) mU/L). We used approximate entropy (ApEn) to quantify the strength of feedback/feedforward control of GH secretion. ApEn was lower (P = 0.001) in offspring (0.45 (0.39–0.53)) compared with controls (0.66 (0.56–0.77)), indicating tighter control of GH secretion. No significant differences were observed in circulating levels of IGF-1 and IGFBP3 between offspring and controls. In conclusion, GH secretion in human familial longevity is characterized by diminished secretion rate and more tight control. These data imply that the highly conserved GH signaling pathway, which has been linked to longevity in animal models, is also associated with human longevity.

INTRODUCTION

Genetic disruption of the insulin/insulin-like growth factor 1 (IGF-1) signaling (IIS) pathway can delay aging and promote longevity in a wide variety of species [1]. In mammalian species, growth hormone (GH) plays a pivotal role in the regulation of the IIS pathway and mutations affecting GH action have consistently been shown to alter lifespan [2]. Increased longevity in mice can be induced by mutations that result in GH deficiency, including the Prop-1 and Pit-1 mutations that cause a combined GH, prolactin, and thyroid-stimulating hormone deficiency, and by deletion of the GH-releasing hormone receptor [3, 4]. Likewise, mutations resulting in GH resistance, notably deletion of the GH receptor, were also found to increase longevity [5]. Accordingly, transgenic mice that overexpress GH are short-lived and show signs of accelerated aging [6]. Also in humans, patients with active acromegaly, who have excessive pituitary GH secretion, were found to have a reduced life expectancy [7]. The results from studies on the association of mutations in the GH pathway that lead to dwarfism in humans with lifespan are contradictory [8]. While Laron syndrome dwarfs with GH receptor gene mutations were found to have relatively long lifespans with reduced risks for cancer and diabetes, patients with untreated GH deficiency had relatively short lifespans [9-11].

However, little is known about how more subtle differences in GH/IGF-1 secretion would affect human longevity. Interestingly, female centenarians were found to be enriched for rare mutations causing slight IGF-1 resistance and resulting in a somewhat smaller stature [12]. Likewise, we previously observed that a combination of polymorphisms in the GH/IIS pathway, linked to smaller stature in female octogenarians, was associated with better survival in old age [13]. However, to the best of our knowledge, no study has assessed the association of human longevity with GH secretion.

GH secretion by somatotrophic cells in the anterior lobe of the pituitary gland is stimulated by growth hormone-releasing hormone (GHRH) and inhibited by somatostatin, both produced by the hypothalamus. GH exerts its functions by binding to GH receptors located on tissue target cells. A key function of GH is to stimulate production of IGF-1 by the liver, which subsequently inhibits GH secretion via negative feedback. Circulating IGF-1 is mostly bound to binding proteins of which insulin-like growth factor binding protein 3 (IGFBP3) is the most abundant. The IGF-1/IGFBP3 molar ratio is considered an indicator of IGF-1 bioavailability. In humans, many other tissues besides the liver express GH receptors indicating that GH may exert effects independent from IGF-1 [14-16]. GH is secreted in a basal (nonpulsatile) and pulsatile mode. The feedback control by IGF-1, together with the feedforward control by GHRH and somatostatin, tightly regulate GH

secretion [17]. The strength of these control signals can be estimated mathematically via calculation of the approximate entropy (ApEn) [18]. Both GH secretion as well as the strength of the feedforward and/or feedback signals were found to be negatively influenced by age and BMI [19, 20]. Interestingly, women had higher 24-h serum GH concentrations and higher ApEn values than men, indicating weaker control of GH secretion [21]. Furthermore, GH secretion is strongly associated with sleep, with the major GH pulse generally occurring shortly after sleep onset [22].

To identify determinants of human longevity, the Leiden Longevity Study (LLS) included offspring of long-lived families that are enriched for exceptional longevity and partners thereof, serving as a control group. Indeed, offspring were found to have less age-related diseases and reduced mortality compared with controls [23]. Previously, no differences were observed between offspring and controls in circulating IGF-1 concentrations [24]. However, the magnitude and control of GH secretion have not yet been studied in human familial longevity. Therefore, we aim in this study to compare GH secretion parameters and the strength of GH secretion control signals between offspring of long-lived families and age-matched controls.

METHODS

Study population

As described previously in more detail, the Leiden Longevity Study (LLS) comprises 421 families with at least two long-lived Caucasian siblings fulfilling the age criteria (men ≥ 89 years and women ≥ 91 years) without selection on health or demographics [23]. In the Switchbox Leiden Study (protocol P11.116), we included 20 offspring of nonagenarian LLS participants together with 18 partners of the offspring as environmental and age-matched controls [25]. All participants were middle-aged (52–76 years), had a stable body mass index (BMI) between 18 and 34 kg/m^2, and women were postmenopausal. Exclusion criteria were having chronic renal, hepatic or endocrine disease, or using medication known to influence lipolysis, thyroid function, glucose metabolism, GH or IGF-1 secretion and/or any other hormonal axis. Moreover, participants were excluded based on presence of fasting plasma glucose > 7 mmol/L, recent trans meridian flight, smoking addiction, or extreme diet therapies. To be able to safely perform the 24-h blood sampling, other exclusion criteria were difficulties to insert and maintain an intravenous catheter, anemia (hemoglobin < 7.1 mmol/L), and blood donation within the last 2 months. Based on information obtained via telephone questioning, controls with a nonagenarian parent who had one or more nonagenarian siblings were also excluded.

The Switchbox Leiden Study was approved by the Medical Ethical Committee of the Leiden University Medical Centre and performed according to the Helsinki declaration. All participants gave written informed consent for participation. For the current study, we excluded one female offspring from the analyses based on a combination of features that suggest a subtle form of GH resistance. Her pulsatile GH secretion (539 mU/L) deviated more than three standard deviations from the mean pulsatile secretion of the women included (193 mU/L), while her height (158 cm) was relatively short compared to the mean height of the women included (165 cm).

Clinical protocol

Full details on the 24-h blood sampling procedure have been described previously [26]. In short, a catheter was placed in a vein of the forearm of the nondominant hand of the participant. Blood sampling started around 09:00 h and every 10 min, 3.2 mL of blood was collected. All participants were sampled in the same research room. The participants received standardized feeding at three fixed times during the day (between 09:00 h and 10:00 h, 12:00 h and 13:00 h and 18:00 h and 19:00 h), each consisting of 600 kcal Nutridrink (Nutricia Advanced Medical Nutrition Zoetermeer, The Netherlands). No naps were allowed during the day, and lights were switched off for approximately 9 h (circa between 23:00 h and 08:00 h). Height and weight were measured in the research center. Body mass index (BMI) was calculated as weight (in kilograms) divided by the square of height (in meters). Waist circumference was measured with a measuring tape midway between the uppermost border of the iliac crest and the lower border of the costal margin.

Assays

All measurements were performed at the Department of Clinical Chemistry and Laboratory Medicine of the Leiden University Medical Centre in The Netherlands, which is accredited according to CCKL (National Coordination Committee for Quality Assurance for Health Care Laboratories in The Netherlands). Laboratory measurements were performed with fully automated equipment and diagnostics from Roche Diagnostics (Almere, The Netherlands) and Siemens Healthcare diagnostics (The Hague, The Netherlands). Human growth hormone (hGH) with a molecular mass of 22 kDa was measured in serum samples using Siemens reagents (catalog number L2KGRH2) and an IMMULITE® 2000 Xpi Immunoassay system (Siemens Healthcare diagnostics). The detection limit was 0.15 mU/L, and the interassay coefficient of variation (CV) ranged between 5.4% at 5.43 mU/L and 7.2% at 25.0 mU/L. The reference range for fasting GH is 0.15–7.25 mU/L. All samples were measured with reagents from the same lot numbers,

and for each participant, all samples (one time series) were measured in the same batch on the same day. IGF-1 (catalog number IS-3900) and IGFBP3 (catalog number IS-4400) were measured in six plasma EDTA samples with 4 h intervals for each participant using an iSYS Immunoassay system of ImmunoDiagnostic Systems (IDS GMBH, Frankfurt am Main, Germany). The CV ranges for IGF-1 and IGFBP3 were 1.4–1.8% and 6.3–7.3%, respectively. Insulin (catalog number L2KIN2) and glucose (catalog number 11876899216) were analyzed in a fasting morning (around 8:30 h) serum sample; insulin (CV ranged between 3.2 and 7.7%) using an IMMULITE® 2000 Xpi Immunoassay system (Siemens Healthcare diagnostics) and glucose (CV ranged between 0.9 and 3.0%) using Hitachi Modular P800 (Roche Diagnostics). In the Netherlands, hGH and IGF-1 test results are routinely harmonized using a national harmonization protocol and a harmonization serum pool as described elsewhere [27]. This means that all hGH and IGF-1 results reported in this study are multiplied with 1.02 and 1.13, respectively.

Deconvolution analysis

The 24-h GH concentration profiles were analyzed by validated deconvolution analysis [28]. By deconvolution analysis, a GH concentration profile is decomposed into underlying secretory bursts, basal secretion, elimination of previously secreted GH and random experimental variability. The algorithm in the software program Matlab (the Mathworks, Inc., Natick, MA) first detrends the data and normalizes concentrations to numbers within the interval 0 to 1. Thereafter, successive potential pulse-time sets, each containing one fewer burst, were created by a smoothing process. Finally, a maximum-likelihood expectation deconvolution method estimated all secretion and elimination rates simultaneously for each candidate pulse-time set. Outcome parameters of main interest are basal (nonpulsatile) secretion, pulsatile secretion, the sum of basal and pulsatile secretion (total secretion), number of pulses per 24 h (secretory-burst frequency), interpulse regularity (Weibull gamma), and slow half-life. Fast half-life was fixed to 3.5 min, and slow half-life was estimated as unknown variable between 8 and 22 min.

Approximate entropy

Approximate entropy (ApEn) is a scale- and model-independent statistic that quantifies the regularity of consecutive time-series data [29]. ApEn has high sensitivity and specificity (both > 90%) for analysis of hormone concentration measurements over 24 h. Low ApEn values imply that the sequence of time-series data is regular and that it contains many repetitive patterns, such as a sinus wave. High ApEn values indicate greater irregularity and randomness. For hormonal data, ApEn provides a direct barometer for the strength of the feedback system. In neuro-endocrine time-series of a length of 50 – 300 data

points, *m* (window length) = 1 is preferred, and for lengths $N \geq 60$, *r* (criterion of similarity) should be set to the predetermined value of 20% of the standard deviation (SD) of the individual subject time series [30]. Therefore, normalized ApEn parameters of $m = 1$ and $r = 20\%$ of the SD of the individual subject time series were used. Additionally, we calculated the Jack-knifed ApEn (JkApEn), which is a rigorous and objective cross-validation test that gives less bias in smaller samples than regular ApEn and it is more applicable for hormone data [31].

Chronotype and sleep

Data on sleep parameters were obtained using the Pittsburgh Sleep Quality Index questionnaire [32]. Chronotype was assessed using the Munich Chronotype questionnaire, with scores ranging from 1 (extreme early type) to 7 (extreme late type) [33, 34].

Statistical analysis

Descriptive statistics were used to summarize the characteristics of study groups. The nonparametric Median Test was used to assess differences between offspring and controls in the variables that were not normally distributed. Independent-samples t-test was used to assess differences between offspring and controls in variables that were normally distributed. GH secretion parameters were compared between offspring and controls using linear regression adjusted for sex and age. Not normally distributed parameters were logarithmic transformed prior to analysis and are presented as geometric means with 95% confidence intervals. $P \leq 0.05$ was considered statistically significant. All statistical analyses were performed with SPSS for Windows, version 20 (SPSS, Chicago, IL, USA). Graphs were made using GraphPad Prism version 6 (GraphPad, San Diego, CA, USA).

RESULTS

Group characteristics

The group characteristics of offspring and controls are presented in Table 1. Participants were selected on the basis of the age of their parents. Consequently, the parents of the offspring were significantly older ($P = 0.01$) than those of the controls. The groups of offspring and controls were similar in age, height, body composition, fasting serum glucose and insulin levels, and sleep parameters. Also the distribution of men and women in both groups was similar.

Table 1. Group characteristics of offspring of long-lived families and controls

	Offspring *n* = 19	Controls *n* = 18	*P* value
Male, *n* (%)	10 (52.6)	10 (55.6)	0.86
Age (years)‡	65.7 (5.6)	64.6 (4.9)	0.52
BMI (kg/m^2)*	24.8 (22.7 – 29.9)	25.1 (22.1 – 27.7)	0.83
Height (cm)*	175 (165 – 180)	175 (167 – 182)	0.73
Waist circumference (cm)*	94 (82 – 101)	94 (83 – 98)	0.75
Fasting glucose [mmol/L]‡	4.9 (0.7)	4.8 (0.4)	0.65
Fasting insulin [mU/L]	4.6 (3.8 – 8.0)	5.9 (3.8 – 7.8)	0.92
Average hours of sleep (h)	7.4 (7.0 – 7.9)	7.4 (6.9 – 7.8)	0.81
Chronotype#	3.0 (2.5 – 3.5)	3.1 (2.6 – 3.6)	0.68
Mean age of parents (years)	88.0 (82.5 – 93.5)	80.3 (74.6 – 84.1)	0.001

Unless indicated otherwise, data are presented as median with interquartile ranges. ‡Data are presented as mean with standard deviation. *Data were not available for one male control subject due to technical problems. #Scores ranging from 1 (extreme early type) to 7 (extreme late type).

GH concentration profiles over 24 h

Individual GH concentration profiles were first assessed by visual inspection. As illustrated in Supplementary Figure S1, which comprises the 24-h GH concentration profiles of all 37 participants, we observed a wide variation between individual 24-h GH profiles. Moreover, due to the pulsatile manner in which GH is secreted, GH concentrations within individual time series vary strongly over 24 h. Therefore, deconvolution analysis was applied to compare specific features of GH secretion between groups.

GH secretion parameters

Table 2 shows that the offspring of long-lived families had a mean (95% CI) total GH secretion over 24 h of 172 (128–216) mU/L. This was significantly lower (P = 0.04) compared with that of controls (238 (193–284) mU/L). The geometric mean (95% CI) basal GH secretion in offspring (14.5 (9.8–21.5) mU/L) was also lower (P = 0.03) compared with that of controls (26.9 (17.9–40.4) mU/L). The pulsatile GH secretion was not significantly different between groups. Results did not change after additional adjustments for BMI or waist circumference (data not shown). Slow half-life, the number of pulses, and the interpulse regularity (Weibull gamma) were similar between offspring and controls. Similar differences as found between offspring and controls were also observed in men and women separately (data not shown).

Table 2. GH secretion parameters in offspring of long-lived families and controls

	Offspring *n* = 19	Controls *n* = 18	*P* value
Slow half-life (min)	16.2 (14.3 – 18.0)	15.2 (13.3 – 17.1)	0.46
Total secretion (mU/L per 24 h)	172 (128 – 216)	238 (193 – 284)	0.04
Basal secretion (mU/L per 24 h)*	14.5 (9.8 – 21.5)	26.9 (17.9 – 40.4)	0.03
Pulsatile secretion (mU/L per 24 h)	151 (113 – 188)	191 (152 – 230)	0.14
Number of pulses (per 24 h)*	13.7 (12.1 – 15.5)	13.8 (12.1 – 15.7)	0.97
Interpulse regularity γ (unitless)	1.3 (1.2 – 1.5)	1.5 (1.3 – 1.7)	0.26

Unless indicated otherwise, data are presented as mean with 95% confidence interval. *Data are presented as geometric mean with 95% confidence interval. Analyzed by linear mixed model adjusted for sex and age.

Approximate entropy

Offspring of long-lived families had a significantly lower (*P* = 0.001) Jack-knifed approximate entropy (JkApEn) than controls (Figure 1). Figure 1 shows the individual JkApEn for all participants with the (unadjusted) mean with standard error. When analyses were adjusted for age and sex, the geometric mean (95% CI) JkApEn was 0.45 (0.39–0.53) for offspring and 0.66 (0.56-0.77) for controls (*P* = 0.001). The differences found between offspring and controls were also observed in men and women separately. Male offspring had a geometric mean (95% CI) JkApEn of 0.38 (0.29–0.48) and male controls of 0.53 (0.42–0.68) (*P* = 0.05). The geometric mean (95% CI) JkApEn of female offspring was significantly lower (*P* = 0.005) than that of female controls (0.53 (0.44–0.65) vs 0.82 (0.67–1.01)). Results did not materially change after additional adjustments for BMI or waist circumference (data not shown).

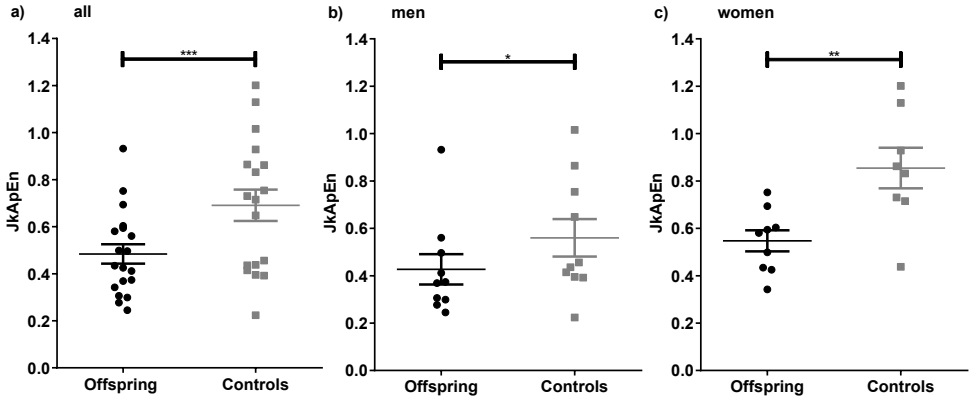

Figure 1. Control of GH secretion in offspring and controls, with lower jack-knifed approximate entropy (JkApEn) indicating tighter control.

JkApEn of GH secretion of offspring and controls in all subjects (A), men (B) and women (C). Lines indicate mean and standard error of the mean. Analyzed by linear mixed model adjusted for sex and age (A) or only age (B and C). $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

IGF-1 and IGFBP3

Geometric mean (95% CI) circulating levels of IGF-1 were 14.2 (12.6–15.9) nmol/L in the offspring and 16.0 (14.2–18.0) nmol/L in controls, which was not significantly different (P = 0.14). Mean IGFBP3 was similar in both groups. The mean (95% CI) IGF-1/IGFBP3 molar ratio was also not significantly different (P = 0.10) in offspring compared with controls (0.128 (0.116–0.140) vs 0.142 (0.130 – 0.155)).

DISCUSSION

The two main findings of this study are that GH secretion is lower and more tightly controlled in subjects enriched for familial longevity compared with age-matched controls.

The observed association between reduced GH secretion and human familial longevity is in line with experimental studies in mice, which found that reduced GH action resulted in extended health and lifespans [3-5]. Our results implicate the highly conserved GH/IGF-1 signaling pathway, which has been linked to delayed aging and longevity in numerous animal models, also in human longevity. The observed differences in GH secretion between offspring and controls can probably not be explained by a faster clearance of GH from the blood, as the slow half-life was comparable between groups. Previous studies found that high approximate entropy (ApEn) values are indicative for reduced strength of feedforward and/or feedback signals on GH secretion. ApEn values were elevated in patients with GH deficiency and in patients with an inactivating defect of the GHRH

receptor gene, compared to healthy individuals [35, 36]. Accordingly, we observed low values of ApEn in the offspring, which are indicative of tighter control.

Various mechanisms may underlie the observed reduction in GH secretion in the offspring, including (i) less stimulatory feedforward drive by GHRH, (ii) increased inhibition by somatostatin, and (iii) increased negative feedback of IGF-1. Veldhuis *et al.* showed that reduced ApEn values can also be explained by any of these three occurrences [18]. Our current data do not allow us to discriminate between these possibilities.

Although somatostatin and GHRH are found in the circulation, these were not measured as it is unknown to what extent these reflect leakage from the brain, or peripheral production. We did measure circulating levels of IGF-1 and IGFBP3, and calculated IGF-1/IGFBP3 molar ratios. The IGF-1/IGFBP3 molar ratio was not higher in the offspring than in the controls. However, other important IGF-1 binding proteins were not measured; thus, we cannot exclude the possibility that differences in IGF-1 bioavailability between groups may exist. Altered GH secretion can also be caused by regulators of GH secretion other than somatostatin, GHRH, and IGF-1. Certain neuropeptides and neurotransmitters also influence GH secretion, such as ghrelin from the stomach, which directly stimulates the release of GH by the pituitary [17, 37].

Different explanations exist for the observed association of diminished GH secretion and familial longevity. A first possibility is that GH is associated with longevity via a reduction of circulating IGF-1. In contrast to long-living model organisms in which besides GH action, IGF-1 levels are reduced, IGF-1 levels were not significantly different between groups, although these tended to be somewhat lower in the offspring. Possibly, our study was underpowered to detect subtle differences in circulating IGF-1 between groups. However, also in a much larger sample of offspring and controls, we previously did not observe differences in circulating IGF-1, nor in stature [24]. Circulating IGF-1 is predominantly produced by the liver. However, many tissues produce paracrine IGF-1 that does not contribute to circulating IGF-1. Interestingly, in Ames dwarf mice with reduced GH secretion, brain IGF-1 levels were elevated compared to control mice [38]. In the current study, data on tissue-specific IGF-1 are lacking. Therefore, the possibility that paracrine IGF-1 is crucial in terms of longevity control could not be addressed. It is also possible that GH is associated with longevity independent of IGF-1. In support of this hypothesis, the impact of disrupting GH signaling on longevity is larger than the impact of disrupting IGF-1 signaling or events downstream from IGF-1 receptors in experimental mice [39]. Also in humans, many tissues besides the liver express GH receptors, and the association

between reduced GH and human familial longevity might be caused by direct effects of GH on these tissues [14-16]. Lastly, it is possible that GH secretion is not causally related to longevity, but rather a pleiotropic side effect of upstream regulators that influence longevity via other mechanisms. For example, in addition to inhibiting GH secretion, somatostatin influences the secretion of other hormones and was found to inhibit cell proliferation and induce apoptosis [40]. Also GHRH was found to have pleotropic effects in the periphery, mainly on tissue regeneration [41].

A strength of our study is that we sampled blood every 10 min during 24 h. Therefore, we could study the somatotropic axis in detail. We performed our study in the LLS, in which families are included based on two proband nonagenarian siblings. A consequence of the LLS study design is that the age range of the offspring varied from 52 to 76 years and GH profiles could not be obtained at young age. Another limitation of this study design is that not every offspring might be enriched for longevity, as possibly not all inherited the favorable predisposition for longevity of their long-lived parent. This could have diluted potential differences between offspring and controls. It is also a limitation that this study was performed with a relatively small sample size.

To conclude, we report for the first time that GH secretion is more tightly controlled in the offspring of long-lived families than in controls. We hypothesize that the offspring are therefore more efficient in regulating the magnitude and the timing of GH secretion. Our data strengthen the hypothesis that GH/IGF-1 signaling is a conserved mechanism implicated in mammalian longevity. We hypothesize that pleiotropic and possibly tissue-specific effects of reduced GH secretion may favor human longevity. Future research should focus on dissecting the mechanisms via which reduced GH is associated with human longevity.

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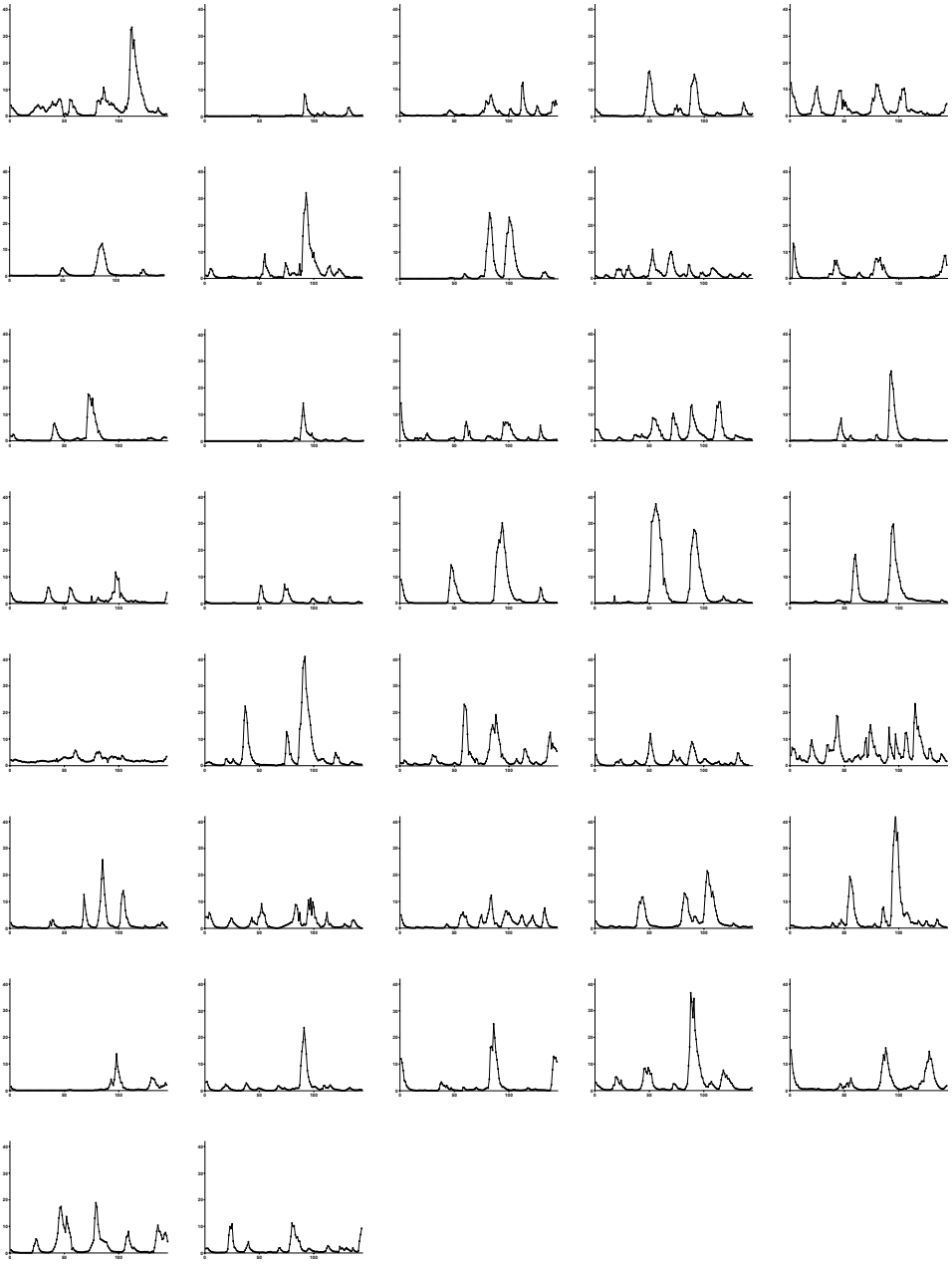
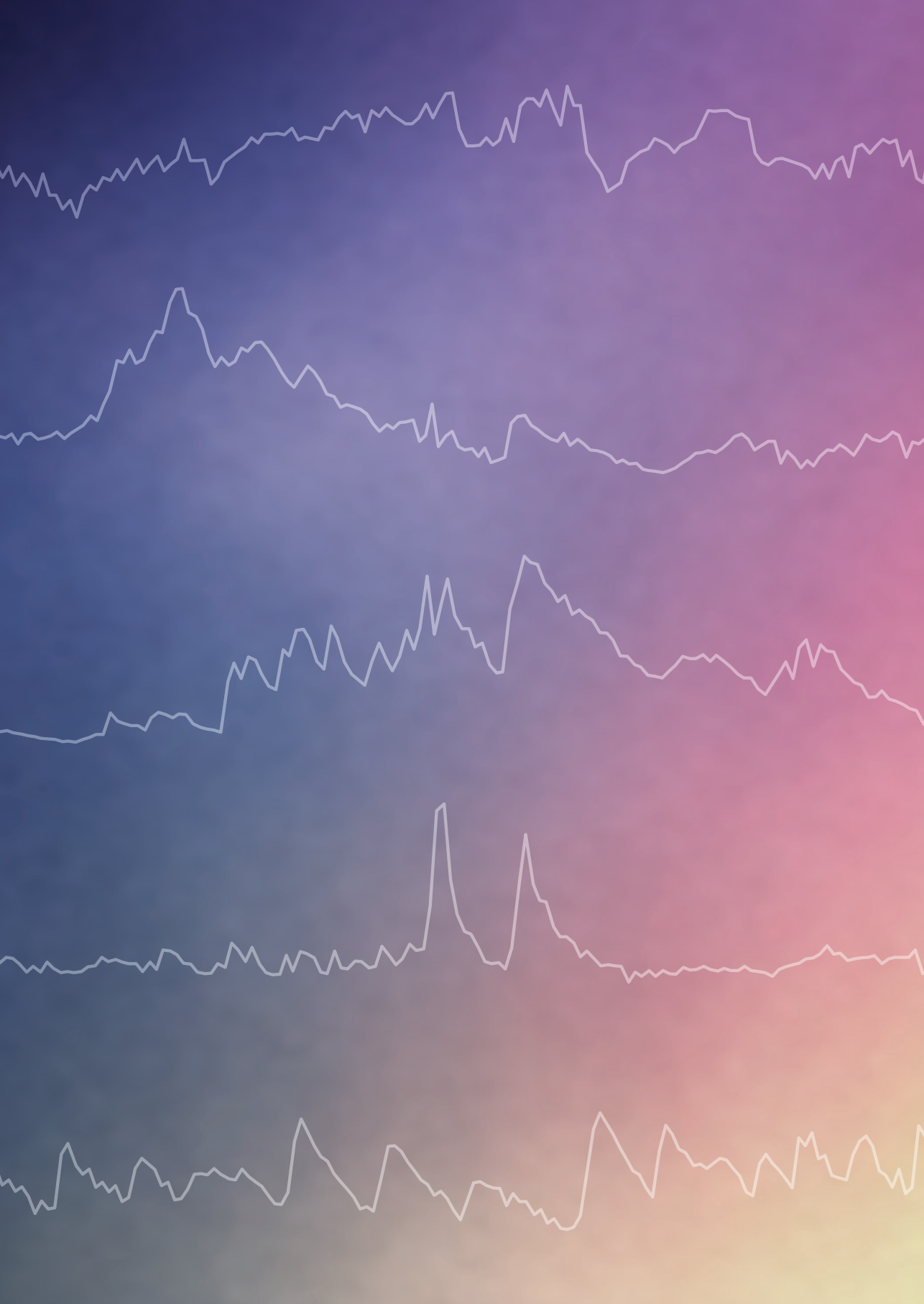
SUPPLEMENTARY DATA



Supplementary Figure S1. 24-hour GH concentration profiles of all 37 participants.
Growth hormone (GH) measurements were performed every 10 minutes for 24 hours, starting around 9:00h. The x-axis presents samplenumber 1 to 144 and the y-axis GH concentration in mU/L.



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Familial longevity is not associated with major differences in the hypothalamic-pituitary-gonadal axis in healthy middle-aged men

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ABSTRACT

Context: A trade-off between fertility and longevity possibly exists. The association of the male hypothalamic-pituitary-gonadal (HPG) axis with familial longevity has not yet been investigated.

Objective: To study 24-h hormone concentration profiles of the HPG axis in men enriched for familial longevity and controls.

Design: We frequently sampled blood over 24 h in 10 healthy middle-aged male offspring of nonagenarian participants from the Leiden Longevity Study together with 10 male age-matched controls. Individual 24-h luteinizing hormone (LH) and testosterone concentration profiles were analysed by deconvolution analyses to estimate secretion parameters. Furthermore, the temporal relationship between LH and testosterone was assessed by cross-correlation analysis. We used (cross-)approximate entropy to quantify the strength of feedback and/or feedforward control of LH and testosterone secretion.

Results: Mean (95% confidence interval (CI)) total LH secretion of the offspring was 212 (156–268) U/L/24 h, which did not differ significantly (P = 0.51) from the total LH secretion of controls (186 (130–242) U/L/24 h). Likewise, mean (95% CI) total testosterone secretion of the offspring (806 (671–941) nmol/L/24 h) and controls (811 (676–947) nmol/L/24 h) were similar (P = 0.95). Other parameters of LH and testosterone secretion were also not significantly different between offspring and controls. The temporal relationship between LH and testosterone and the strength of feedforward/feedback regulation within the HPG axis were similar between offspring of long-lived families and controls.

Conclusions: This relatively small study suggests that in healthy male middle-aged participants, familial longevity is not associated with major differences in the HPG axis. Selection on both fertility and health may in part explain the results.

INTRODUCTION

Over the last decades, several conserved mechanisms have been identified that associate with longevity in both animal models and humans. The common function of these evolutionarily conserved systems is to enable the organism to adequately respond to changes in the environment in order to maintain homeostasis. This is achieved by adapting the balance between growth, development, and reproduction versus maintenance and repair [1]. In mammals, these adaptive responses are centrally regulated and involve the hypothalamus. The hypothalamus regulates homeostasis via neural and endocrine pathways. These latter comprise the hypothalamic-pituitary-thyroid (HPT) axis, the hypothalamic-pituitary-growth hormone (GH) axis, the hypothalamic-pituitary-adrenal (HPA) axis, the hypothalamic-pituitary-prolactin (PRL) axis, and the hypothalamic-pituitary-gonadal (HPG) axis. To maintain homeostasis, these centrally regulated hormonal axes respond to stressors, such as inflammation and starvation. As a response to systemic inflammatory disease, the HPA axis is activated, and available resources are re-allocated by suppressing the GH, HPG, and HPT axes among others [2]. Likewise, fasting was observed to suppress the HPG, HPT, GH, and PRL axes, and to stimulate the HPA axis in healthy men [3]. While these adaptive responses are clearly beneficial for short term survival, their long-term health consequences may vary depending on the type and severity of the stress. Extension of health and lifespan can also be induced by genetic mutations altering one or more neuroendocrine axes [4, 5]. In model organisms, many long-lived mutants have a reduced reproductive output. Also in humans, decreased reproduction was found to associate with exceptional human longevity in both men and women [6].

It is well known that the lifespan of men is shorter than that of women. It has been speculated that enhanced exposure to male sex hormones and/or decreased exposure to female sex hormones may be contributing factors. Oestrogens in women were found to upregulate the expression of antioxidant, longevity-related genes, which could be a biological explanation for sex differences in longevity [7]. In line, a later age of menopause was associated with reduced female mortality [8]. In men, the HPG axis involves hypothalamic gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH) from gonadotrophs in the anterior lobe of the pituitary gland and testosterone from the Leydig cells of the testis. Little is known about direct effects of the male HPG axis on human longevity. Studies in castrated men are inconclusive; Korean eunuchs had a longer lifespan than non-castrated men of similar socioeconomic status, whereas the lifespan of castrated singers was similar to that of non-castrated singers [9, 10]. Longevity inducing interventions, such as fasting, lead to a decline in LH secretory burst mass and

testosterone concentrations and to an increase in LH release pattern orderliness [3, 11]. However, the association of reduced testosterone secretion or other characteristics of the male HPG axis with human familial longevity has not been investigated yet.

In order to identify determinants of human longevity, the Leiden Longevity Study (LLS) included offspring of long-lived families that are enriched for exceptional longevity and partners thereof, serving as a control group [12]. To date, the 24-h profiles of three hypothalamic hormonal axes have been investigated in detail in the LLS. It was found that TSH secretion was higher and TSH-fT3 temporal relationship was stronger in the offspring compared to controls [13, 14]. Furthermore, we found that GH secretion was diminished and tightly controlled in offspring compared to controls [15]. However, we did not find an association between familial longevity and cortisol secretion under resting conditions [16]. In the current study, we test whether male sex hormones are associated with familial longevity by comparing 24-h LH and testosterone secretion parameters, the temporal relationship between LH and testosterone, and the strength of feedforward and feedback control signals within the HPG axis between male offspring of long-lived families and age-matched controls.

METHODS

Study population

The LLS comprises 421 families with at least two long-lived Caucasian siblings fulfilling the age criteria (men ≥ 89 years and women ≥ 91 years) without selection on health or demographics, as described previously in more detail [12]. In the Switchbox Leiden Study, we included 20 offspring of nonagenarian LLS participants together with 18 partners of the offspring as environmental- and age-matched controls [13]. Exclusion criteria were having chronic renal, hepatic or endocrine disease, or using medication known to influence lipolysis, thyroid function, glucose metabolism, GH or IGF-1 secretion, and/or any other hormonal axis. Moreover, participants were excluded based on the presence of fasting plasma glucose > 7 mmol/L, recent trans meridian flight, smoking addiction, or extreme diet therapies. To be able to safely perform the 24-h blood sampling, other exclusion criteria were difficulties to insert and maintain an intravenous catheter, anaemia (haemoglobin < 7.1 mmol/L), and blood donation within the last 2 months. Based on information obtained via telephone questioning, controls with a nonagenarian parent who had one or more nonagenarian siblings were also excluded. Participants were middle-aged (52–76 years) and had a stable body mass index (BMI) between 18 and 34 kg/m^2. The Switchbox Leiden Study was approved by the Medical Ethical Committee of the

Leiden University Medical Centre and was performed according to the Helsinki declaration. All participants gave written informed consent for participation.

Clinical protocol

Full details on the 24-h blood sampling procedure have been described previously [17]. In short, a catheter was placed in a vein of the forearm of the non-dominant hand and, starting around 09:00 h, 3.2 mL of blood was collected every 10 min. Participants received standardized feeding consisting of 600 kcal Nutridrink (Nutricia Advanced Medical Nutrition Zoetermeer, The Netherlands) at three fixed times during the day. Participants were not allowed to sleep during the day and except for lavatory use no physical activity was allowed during the study period. Lights were switched off for approximately 9 h (circa between 23:00 and 08:00 h) to allow the participants to sleep. Height and weight were measured in the research centre. BMI was calculated as weight (in kilograms) divided by the square of height (in meters). Body composition was determined by Bioelectrical Impedance Analysis (BIA) at a fixed frequency of 50 kHz (Bodystat® 1500 Ltd, Isle of Man, British Isles) [18]. Waist circumference was measured with a measuring tape midway between the uppermost border of the iliac crest and the lower border of the costal margin.

Hormone assays

All hormonal assays were performed with fully automated equipment and diagnostics from Roche Diagnostics (Almere, The Netherlands) at the Department of Clinical Chemistry and Laboratory Medicine of the Leiden University Medical Centre in The Netherlands, which is accredited according to the National Coordination Committee for Quality Assurance for Health Care Laboratories in The Netherlands. LH (catalogue number 11732234122) and testosterone (catalogue number 05200067190) were measured in EDTA plasma samples collected every 10 min using ECLIA (Electrochemoluminiscentie immunoassay) on a Roche Modular E170 immunoanalyser. The measuring range of LH is 0.100-200 U/L and the interassay coefficients of variation (CV) were 4.47% at 4.10 U/L and 2.83% at 56.43 U/L. The measuring range of testosterone is 0.087-52.0 nmol/L and the interassay CV were 4.12% at 1.77 nmol/L and 3.78% at 34.84 nmol/L. Testosterone is mostly bound to binding proteins including sex hormone-binding globulin (SHBG) and albumin. Albumin (catalogue number 11970909216) and SHBG (catalogue number 03052001190) were measured on Roche Modular analysers in six EDTA plasma samples with 4-h intervals for each participant. Free testosterone was calculated based on albumin and SHBG levels as described by Vermeulen *et al.* [19].

Additional blood measurements

Approximately 2 weeks before the study day, fasting serum was withdrawn to screen for factors that might have an impact on testosterone production. Dehydroepiandrosterone sulphate (DHEAS) with catalogue number L2KDS2 was measured using a solid-phase competitive chemoluminescent enzyme immunoassay with an Immulite 2000 XPi system from Siemens Healthcare diagnostics (The Hague, The Netherlands). Total 25-hydroxyvitamin D (catalogue number 05894913190) was measured using ECLIA with an E170 module of Modular Analytics from Roche Diagnostics. Interleukin 6 (catalogue number SS600B) and tumour necrosis factor-α (TNF-α) (catalogue number SSTA00D) were measured by ELISA from R&D Systems. High-sensitivity C reactive protein (hsCRP) with catalogue number 04628918190 was determined by a particle-enhanced turbidimetric assay using Cobas Integra 800 from Roche Diagnostics.

24-h pool measurements

For each participant, 3 µl of all (144) samples taken during the 24-h blood sampling were pooled. In this mixture, the levels of testosterone (catalogue number 05200067190), oestradiol (catalogue number 06656021190), and prolactin (catalogue number 03203093190) were determined using ECLIA and E170 module of Modular Analytics from Roche Diagnostics.

Deconvolution analysis

To determine underlying components of LH and testosterone secretion, 24-h LH and testosterone concentration profiles were analysed by validated deconvolution analysis [20]. By deconvolution analysis, a hormone concentration profile is decomposed into underlying secretory bursts, basal secretion, elimination of previously secreted hormone and random experimental variability. The algorithm in the Matlab software program (Mathworks, Inc., Natick, MA, USA) first detrends the data and normalizes concentrations to numbers within the interval 0-1. Thereafter, successive potential pulse-time sets, each containing one fewer burst, were created by a smoothing process. Finally, a maximum-likelihood expectation deconvolution method estimated all secretion and elimination rates simultaneously for each candidate pulse-time set. Outcome parameters of main interest are basal (non-pulsatile) secretion, pulsatile secretion, the sum of basal and pulsatile secretion (total secretion), number of pulses per 24 h (secretory burst frequency), interpulse regularity (Weibull gamma), mean pulse mass, and slow half-life. For LH, fast half-life was fixed to 6.93 min and slow half-life was estimated as unknown variable between 40 and 120 min [21]. For testosterone, fast and slow half-lives were fixed to 1.4 and 27 min, respectively [22].

Approximate entropy

Jack-knifed approximate entropy (JkApEn) is a measure for the strength of feedforward and feedback control signals in a hormone system. First, approximate entropy (ApEn), which is a scale- and model-independent statistic that quantifies the regularity of consecutive time-series data, was calculated using the Matlab software program (Mathworks, Inc., Natick, MA, USA) [23]. ApEn has high sensitivity and specificity (both > 90%) for analysis of hormone concentration measurements over 24 h. Low ApEn values imply that the sequence of time-series data is regular and that it contains many repetitive patterns. High ApEn values indicate greater irregularity and randomness. Normalized ApEn parameters of *m* (window length) = 1 and *r* (criterion of similarity) = 20% of the SD of the individual subject time series were used [24]. Subsequently, jack-knifing was performed, which is a rigorous and objective leave-one-out cross validation test that gives less bias in smaller samples than regular ApEn, and it is more applicable for hormone data [25].

Cross-approximate entropy

Bivariate cross-ApEn is a scale- and model-independent regularity statistic, which quantifies the relative pattern synchrony of two time series [26]. Changes in the cross-ApEn reflect feedback and/or feedforward alterations within an interlinked axis, with the cross-ApEn of LH-T representing feedforward asynchrony and cross-ApEn of T-LH indicating feedback asynchrony [27].

LH/T and T/LH ratios

Besides ApEn, other proxies for the strength of the feedforward and feedback signalling in the HPG axis are the ratios of LH and testosterone concentrations. LH/testosterone ratio reflects the feedback of testosterone on LH secretion, with a higher ratio signifying lower feedback. Testosterone/LH ratio reflects the strength of the feedforward drive of LH on the testosterone secretion, with a higher ratio denoting higher feedforward [28].

Cross-correlation

The temporal relationship between LH and testosterone concentrations was determined by cross-correlation. Cross-correlation assesses the relative strength between a hormone pair for all possible time shifts. For the offspring and control group, the strongest correlation coefficient with corresponding lag time were compared.

Statistical analysis

Descriptive statistics was used to summarize the characteristics of study groups. The non-parametric Mann-Whitney U-test and independent-samples T-test were used to assess

differences between offspring and controls in, respectively, variables that were not normally distributed and normally distributed variables. LH and testosterone secretion parameters were compared between offspring and controls using linear regression adjusted for age. Variables were examined for normality. Variables that were not normally distributed were logarithmically transformed prior to analysis and are presented as geometric means with 95% confidence intervals (CIs), except for two variables, LH slow half-life and testosterone total secretion, because logarithmic transformation did not improve normality. P≤0.05 was considered statistically significant. All statistical analyses were performed using the SPSS for Windows, version 23 (SPSS, Chicago, IL, USA). Graphs were made using GraphPad Prism version 6 (GraphPad, San Diego, CA, USA).

RESULTS

Group characteristics

Group characteristics of male offspring of long-lived families and controls are presented in Table 1. Participants were selected based on the age of one of their parents. Consequently, the parents of the offspring were significantly older on average (P = 0.02) than those of the controls. Offspring and controls were similar in age, body composition, available immunological markers, DHEAS, and 25-hydroxyvitamin D. Group characteristics of female offspring of long-lived families and controls are presented in Table S1. Similarly to men, female offspring and controls did not differ significantly in age, body composition, available immunological markers, DHEAS, and 25-hydroxyvitamin D, except for mean age of the parents.

Table 1. Group characteristics of male offspring of long-lived families and controls

	Offspring *n* = 10	**Controls *n* = 10**	***P* value**
Age (years)[‡]	66.6 (6.4)	64.6 (4.0)	0.41
BMI (kg/m^2)[‡*]	26.0 (3.4)	25.7 (3.2)	0.84
Height (cm)[*]	177 (175 – 182)	181 (175 – 184)	0.60
Fat mass (kg)[*]	19.8 (16.4 – 25.2)	18.5 (18.1 – 23.6)	0.84
Lean body mass (kg)[*]	61.8 (56.5 – 66.2)	60.5 (58.3 – 66.3)	0.72
Waist circumference (cm)[*]	96.5 (88.3 – 109.8)	97.0 (93.0 – 104.0)	0.91
Mean age of parents (years)	89.0 (83.4 – 95.0)	79.5 (74.6 – 84.3)	0.02
DHEAS [µmol/L]	4.0 (1.5 – 5.9)	2.6 (2.0 – 6.4)	0.74
Vitamin D [nmol/L]	71.9 (53.0 – 103.0)	69.9 (56.5 – 85.6)	0.91
Interleukin 6 [pg/ml]	0.8 (0.6 – 1.3)	1.1 (0.9 – 1.6)	0.22
TNF-α [pg/ml]	1.7 (1.4 – 4.0)	1.4 (1.3 – 2.2)	0.22
hsCRP [mg/L][#]	0.7 (0.6 – 3.0)	0.9 (0.6 – 1.2)	0.83

Unless indicated otherwise, data are presented as median with interquartile ranges. Bold values indicate $P \leq 0.05$. [‡]Data are presented as mean with SD. [*]Data were not available for one control subject due to technical problems. [#]Two controls were excluded with hsCRP > 20 (indicative of acute inflammation) approximately 2 weeks before the study day.

LH and testosterone secretion

To illustrate the wide variation between participants in LH and testosterone concentration profiles, Figures S1 and S2 present the individual 24-h LH and testosterone concentration profiles of all participants, respectively. Results of the deconvolution analyses on 24-h concentration profiles of plasma LH and testosterone are shown in Table 2. Mean (95% CI) total LH secretion of the offspring was 212 (156 – 268) U/L/24 h and that of controls 186 (130 – 242) U/L/24 h, which did not differ significantly ($P = 0.51$). Likewise, basal LH secretion, pulsatile LH secretion, and characteristics of pulsatile LH secretion were similar between the groups. Mean (95% CI) total testosterone secretion of the offspring was 806 (671 – 941) nmol/L/24 h, which was similar ($P = 0.95$) to the mean (95% CI) total testosterone secretion of controls (811 (676 – 947) nmol/L/24 h). Other parameters of testosterone secretion were also not significantly different between the offspring and controls.

Table 2. LH and testosterone secretion parameters in male offspring of long-lived families and controls

	Offspring *n* = 10	Controls *n* = 10	*P* value
LH			
Mean LH [U/L]	5.1 (3.9 – 6.4)	5.3 (4.0 – 6.5)	0.89
Slow half-life (min)‡	59.7 (46.6 – 72.8)	69.9 (56.7 – 83.0)	0.27
Total secretion (U/L per 24 h)	212 (156 – 268)	186 (130 – 242)	0.51
Basal secretion (U/L per 24 h)	134 (94 – 175)	116 (76 – 156)	0.51
Pulsatile secretion (U/L per 24 h)*	69.9 (51.8 – 94.3)	64.5 (47.8 – 87.0)	0.70
Number of pulses (per 24 h)	14.4 (13.1 – 15.7)	13.7 (12.4 – 15.0)	0.46
Interpulse regularity γ*	2.2 (1.9 - 2.6)	2.2 (1.9 - 2.7)	0.88
Mean pulse mass [U/L]	5.3 (4.0 - 6.5)	5.1 (3.9 - 6.4)	0.85
Testosterone			
Mean testosterone [nmol/L]*	14.2 (12.1 - 16.8)	13.8 (11.7 - 16.2)	0.78
Mean calculated free testosterone [nmol/L]*	0.43 (0.38 – 0.49)	0.40 (0.35 – 0.45)	0.39
Total secretion (nmol/L per 24 h)‡	806 (671 – 941)	811 (676 – 947)	0.95
Basal secretion (nmol/L per 24 h)	608 (502 – 714)	619 (513 – 726)	0.88
Pulsatile secretion (nmol/L per 24 h)	198 (138 – 258)	192 (132 – 252)	0.88
Number of pulses (per 24 h)	23.0 (19.3 – 26.6)	21.7 (18.1 – 25.4)	0.62
Interpulse regularity γ	2.6 (2.1 – 3.1)	2.3 (1.9 – 2.8)	0.44
Mean pulse mass [nmol /L]	8.3 (6.3 – 10.2)	8.8 (6.8 – 10.8)	0.69

Unless indicated otherwise, data are presented as mean with 95% confidence interval and analysed by linear regression adjusted for age. *Data are presented as geometric mean with 95% confidence interval. ‡Results of the Mann-Whitney U-tests were similar to results of linear regression.

Feedforward and feedback regulation of LH and testosterone secretion

Approximate entropy of LH and of testosterone, representing the strength of feedback and feedforward regulation, were similar between offspring and controls, as shown in Table 3. LH-T cross-ApEn, reflecting feedforward synchrony, was similar between offspring of long-lived families and controls. Equally, T-LH cross-ApEn, reflecting feedback synchrony, was not significantly different between the groups. Furthermore, mean LH/T ratio, representing feedforward drive, and mean T/LH ratio, representing feedback, were similar between offspring and controls.

Table 3. Proxies of feedforward and feedback regulation of LH and testosterone (T) secretion in male offspring of long-lived families and controls

	Offspring *n* = 10	Controls *n* = 10	*P* value
LH ApEn	1.5 (1.3 – 1.7)	1.4 (1.2 – 1.6)	0.54
T ApEn	1.8 (1.6 – 1.9)	1.6 (1.4 – 1.7)	0.11
LH-T cross-ApEn (feedforward asynchrony)	1.8 (1.6 – 1.9)	1.6 (1.4 – 1.8)	0.40
T-LH cross-ApEn (feedback asynchrony)	2.0 (1.8 – 2.2)	1.8 (1.6 – 2.0)	0.25
Mean LH/T ratio* (feedback)	0.3 (0.3 - 0.5)	0.4 (0.3 - 0.5)	0.75
Mean T/LH ratio (feedforward)	3.6 (2.6 – 4.6)	3.1 (2.2 – 4.1)	0.50

Unless indicated otherwise, data are presented as mean with 95% confidence interval. *Data are presented as geometric mean with 95% confidence interval. Analyses are adjusted for age.

Temporal relationship of LH and testosterone

The maximal correlation between LH and testosterone concentrations was found at a lag time between 60 and 80 min with a correlation coefficient (*r*) of 0.16 for offspring and *r* = 0.29 for controls, which was not significant different (*P* = 0.21) when adjusted for age.

Measurements in 24-h pool

Because all women were postmenopausal, determination of sex hormones was limited to a single measurement in the 24-h pool. No differences were observed in testosterone, oestradiol, and prolactin levels between offspring and controls, neither in men nor in women (Table 4).

Table 4. Testosterone, oestradiol, and prolactin in a 24-h pool for offspring of long-lived families and controls

	Men			Women		
	Offspring *n* = 10	Controls *n* = 10	*P* value	Offspring *n* = 10	Controls *n* = 8	*P* value
24-h testosterone [nmol/L]	15.4 (12.1 – 18.9)	14.3 (12.2 – 18.0)	0.85	0.47 (0.35 – 1.14)	0.31 (0.16 – 0.49)	0.24
24-h oestradiol [pmol/L]	81.7 (60.5 – 97.2)	83.2 (66.2 – 96.4)	0.99	15.3 (9.2 – 37.7)	9.2 (9.2 – 27.7)	0.57
24-h prolactin [µg/L]	8.9 (7.8 – 9.5)	8.4 (7.4 – 9.7)	0.63	10.4 (8.5 – 12.8)	10.7 (9.6 – 13.8)	0.76

Data are presented as median with interquartile ranges and analysed with Mann-Whitney U-tests.

DISCUSSION

In this study, we did not observe significant differences in 24-h secretion of HPG axis parameters and their regulation between male subjects enriched for familial longevity compared to controls. LH and testosterone secretion parameters, and the strength of feedforward and feedback regulation within the HPG axis, were similar between offspring of long-lived families and controls. Also, testosterone, oestradiol, and prolactin levels measured in a 24-h pool were similar between the offspring and controls in men and in women.

According to the disposable soma theory, which suggests a trade-off between fertility and longevity, longevity would be associated with reduced reproductive capacity, i.e. reduced HPG axis action [1]. However, we observed in the current study that male offspring of long-lived families did not differ from age-matched controls in their HPG axis. There are several potential explanations for the observed absence of an association between male HPG axis and familial longevity.

Possibly, this observation is related to the selection criteria of the study population. First, due to the recruitment of the study population based on the presence of at least two long-lived siblings, we may have selected on fertility, which is strongly associated with the HPG axis. Second, we applied strict exclusion criteria based on health. Health is highly associated with the HPG axis; health status can influence the HPG axis and conversely, the HPG axis can influence health. Inflammation is a major factor influencing the HPG axis, which was also shown recently by Veldhuis *et al.* reporting on healthy male subjects receiving an IL-2 injection which led to an immediate decrease in testosterone secretion [28]. Even though it is widely accepted that starting at the age of around 30-40 years, testosterone levels decline relatively linearly with age, some studies have shown that testosterone does not significantly decline with age in exceptionally healthy men [29-31]. It is unclear whether age-related pathophysiological features, which are associated with a decline in testosterone, are a consequence of testosterone decline or partly the cause. However, testosterone supplementation can reverse some of these pathophysiological features, indicating that testosterone itself has a major influence on age-related pathophysiology [32]. Because of the strict exclusion criteria on health, the two groups of men were very similar in their health status, as also indicated by similar markers of health, and possibly consequently also in their HPG axis.

Supporting our findings, no mutations in the HPG axis have been associated with changes in lifespan in model organisms, indicating that a constitutively lower activity of the HPG

axis is likely not associated with longevity. Long-living IGF-1 and GH-mutant mice do show suppression in HPG hormones and reproduction, but mainly in female mice [33]. However, suppression of HPG hormones and reproduction might not be essential for their longevity phenotype, but rather be a pleiotropic effect of reduced GH/IGF-1 signalling. Moreover, the possible trade-off between fertility and longevity could be only present in women and not in men. This could be explained by the fact that the amount of energy invested in reproduction is much higher in women than in men. However, the possible trade-off in women seems not to be linear, but U-shaped, with an optimum of having approximately two children [34]. In the current study, we did not find an association between sex hormones and familial longevity in women. However, we were limited to a single measurement in postmenopausal women. Thus, testing this hypothesis should preferably be performed in women with a younger age. Finally, the HPG axis might be too important for fitness, thus preventing the spread of mutations that would constitutively downregulate this axis. It has even been argued that because men are able to reproduce up to high ages, late-life male reproductive success would drive a positive association between fertility and longevity [35].

One of the limitations of the current study was the relatively small sample size. This study is underpowered to detect many small differences but adequately sampled to identify major differences in hormonal axes. Indeed, in the same study population, comprising men and women, offspring had a 59.9% higher total TSH secretion and a higher temporal correlation between TSH and fT3 compared to controls [13, 14]. Furthermore, GH secretion was lower and tighter controlled in the offspring compared to controls [15]. When restricted to men only, which is a sample identical to the sample of men used in the current study, differences of similar magnitude were found in TSH secretion, temporal TSH-fT3 correlation, GH secretion, and GH ApEn.

Inherent to the study design of the LLS, in which families are included based on two proband nonagenarian siblings, the age range of the offspring was 52-76 years. Therefore, LH and testosterone profiles could not be obtained at young age. Another limitation of this study was that there could have been dilution of potential differences between offspring and controls, since possibly not all offspring may have inherited the favourable predisposition for longevity of their long-lived parent. Strength of our study is that we sampled blood every 10 min during 24 h, creating the opportunity to study the HPG axis in detail. Moreover, we performed our study in a special cohort, in which we were able to study human familial longevity.

To conclude, this relatively small study suggests that in healthy male middle-aged participants, human familial longevity is not associated with major differences in the HPG axis.

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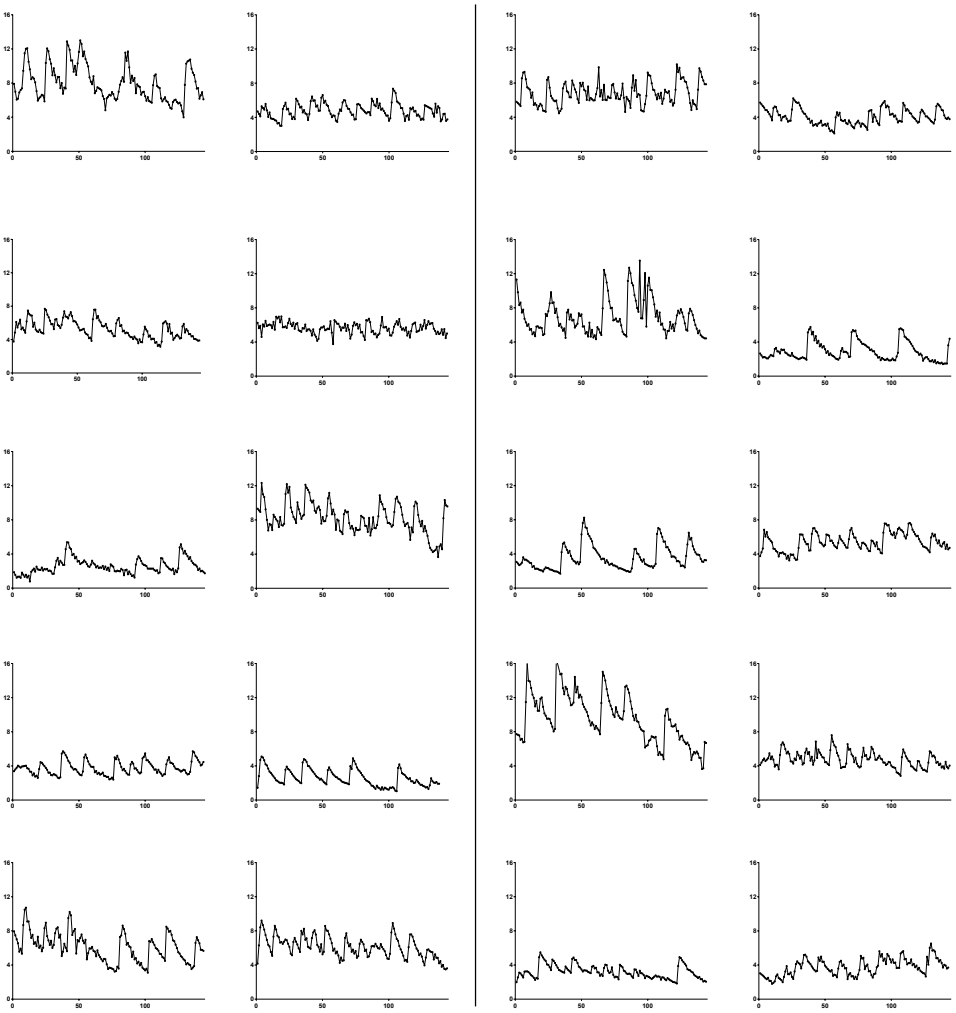
SUPPLEMENTARY DATA

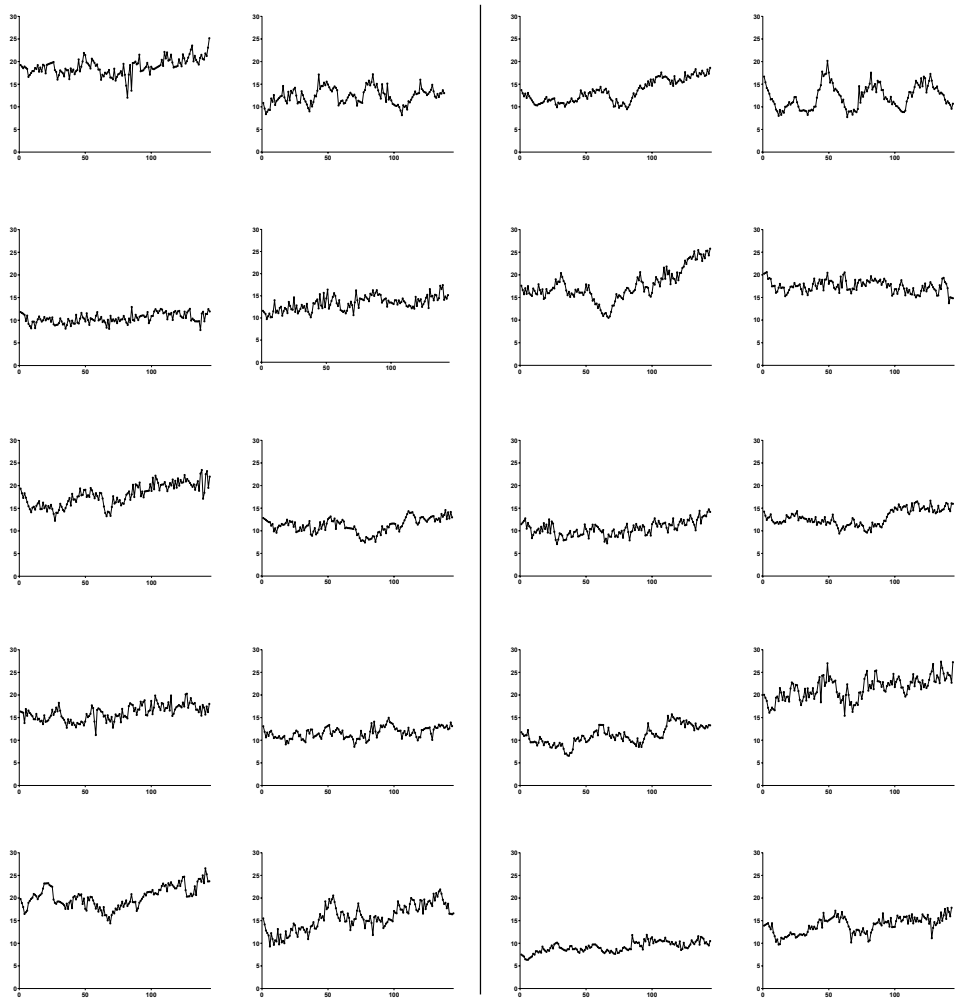
Supplementary Table S1. Group characteristics of female offspring of long-lived families and controls

	Offspring *n* = 10	**Controls *n* = 8**	***P* value**
Age (years)‡	64.7 (4.4)	64.5 (6.1)	0.95
BMI (kg/m^2)	23.5 (21.6 – 30.6)	23.1 (21.1 – 28.9)	0.99
Height (cm)	164 (161 – 168)	167 (161 – 171)	0.57
Fat mass (kg)	23.5 (19.7 – 34.7)	23.5 (19.1 – 34.6)	0.97
Lean body mass (kg)	42.0 (36.8 – 44.7)	40.4 (37.7 – 47.4)	0.90
Waist circumference (cm)	82.0 (80.3 – 100.3)	86.5 (80.0 – 94.8)	0.83
Mean age of parents (years)	90.3 (82.3 – 93.1)	80.5 (72.3 – 84.0)	0.02
DHEAS [µmol/L]*	3.0 (1.8 – 4.7)	1.8 (1.2 – 2.4)	0.07
Vitamin D [nmol/L]*	74.0 (61.4 – 115.3)	85.1 (60.9 – 101.5)	0.96
Interleukin 6 [pg/ml]*	1.1 (0.8 – 2.1)	1.2 (1.0 – 1.8)	0.74
TNF-α [pg/ml]*	1.6 (1.4 – 6.1)	2.0 (1.4 – 4.4)	0.89
hsCRP [mg/L]*	1.1 (0.6 – 3.6)	1.2 (0.7 – 2.0)	0.99

Unless indicated otherwise, data are presented as median with interquartile ranges. ‡Data are presented as mean with standard deviation. *Data were not available for one offspring.

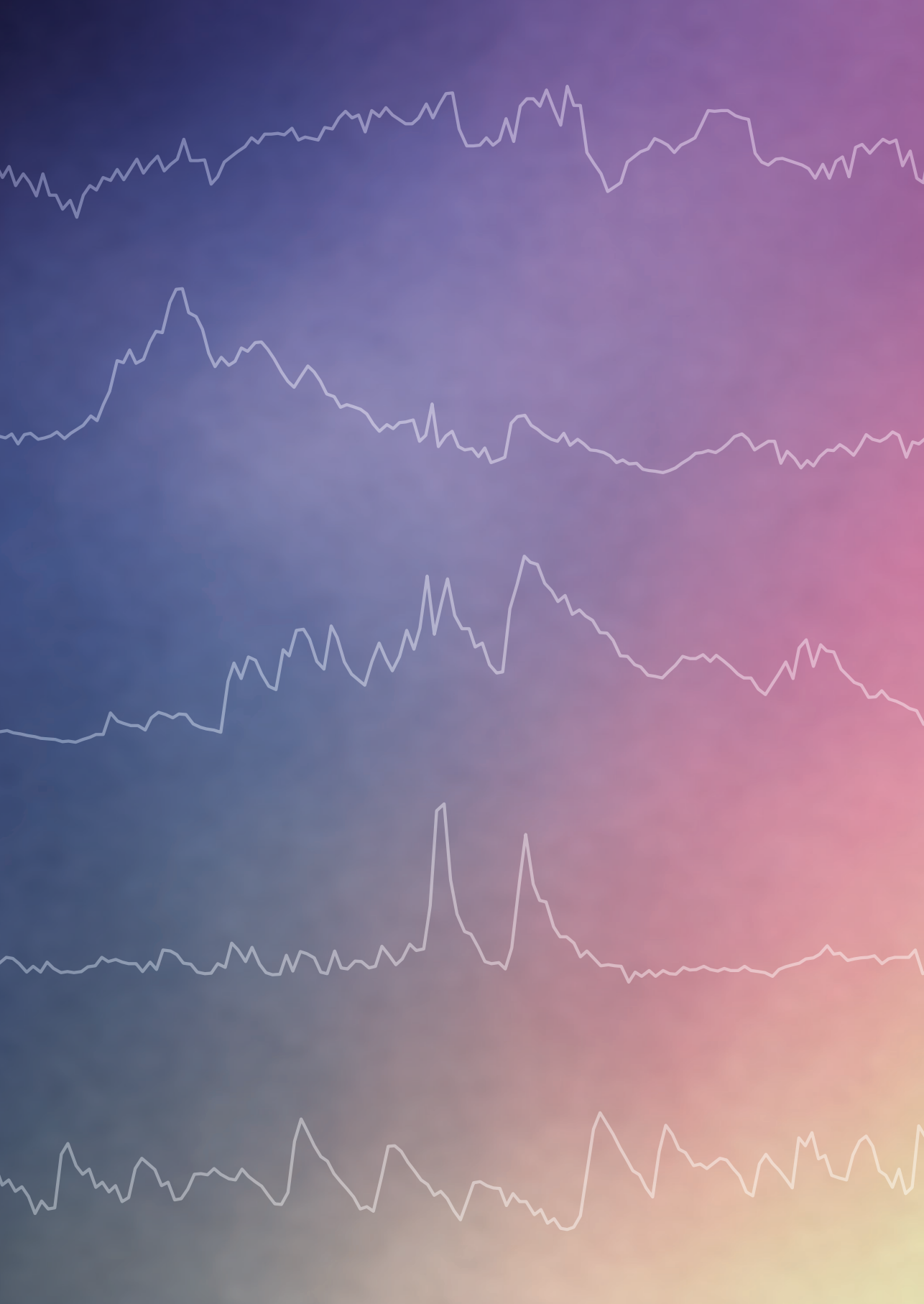


Supplementary Figure S1. 24-hour LH concentration profiles of all participants.
Left columns present luteinizing hormone (LH) concentration profiles of 10 offspring of long-lived families and right columns of 10 controls. LH measurements were performed every 10 minutes for 24 hours, starting around 9:00 h. The x-axis presents sample number 1 to 144 and the y-axis LH concentration in U/L.



Supplementary Figure S2. 24-hour testosterone concentration profiles of all participants.

Left columns present testosterone concentration profiles of 10 offspring of long-lived families and right columns of 10 controls. Testosterone measurements were performed every 10 minutes for 24 hours, starting around 9:00 h. The x-axis presents sample number 1 to 144 and the y-axis testosterone concentration in nmol/L.



5

Interrelationships between pituitary hormones as assessed from 24-h serum concentrations in healthy older subjects

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ABSTRACT

Context: Hormones of the hypothalamic-pituitary-target gland axes are mostly investigated separately, while the interplay between hormones might be as important as each separate hormonal axis.

Objective: Our aim is to determine the interrelationships between GH, TSH, ACTH, and cortisol in healthy older individuals.

Design: We made use of 24-h hormone serum concentrations assessed with intervals of 10 min from 38 healthy older individuals with a mean age (SD) of 65.1 (5.1) years from the Leiden Longevity Study. Cross-correlation analyses were performed to assess the relative strength between two 24-h hormone serum concentration series for all possible time shifts. Cross-approximate entropy was used to assess pattern synchronicity between two 24-h hormone serum concentration series.

Results: Within an interlinked hormonal axis, ACTH and cortisol were positively correlated with a mean (95% CI) correlation coefficient of 0.78 (0.74 – 0.81) with cortisol following ACTH concentrations with a delay of 10 min. Between different hormonal axes, we observed a negative correlation coefficient between cortisol and TSH of -0.30 (-0.36 – -0.25) with TSH following cortisol concentrations with a delay of 170 min. Furthermore, a positive mean (95% CI) correlation coefficient of 0.29 (0.22 – 0.37) was found between TSH and GH concentrations without any delay. Moreover, cross-ApEn analyses showed that GH and cortisol exhibit synchronous serum concentration patterns.

Conclusions: This study demonstrates that interrelations between hormones from interlinked as well as different hypothalamic-pituitary-target gland axes are observed in healthy older individuals. More research is needed to determine the biological meaning and clinical consequences of these observations.

INTRODUCTION

Hormones of the hypothalamic-pituitary-target gland axes are regulated by central and peripheral feedforward and feedback signals. The interplay among these regulators in time dictates the hormone secretion pattern, which will be adapted depending on the changing needs of the organism, such as during the circadian rhythm, sleep, activity, food intake, stress, and inflammation. Although hormones of the hypothalamic-pituitary-target gland axes are each regulated by different factors and respond to different signals, the common goal of all these hormonal axes is to maintain homeostasis in the human body. Furthermore, anterior pituitary cells share the same embryonic origin – the anterior pituitary is derived from oral ectoderm – and pituitary hormones carry out their actions in similar ways [1]. Moreover, there is evidence for crosstalk between pituitary cells [2]; studies in rats showed that there is functional overlap between the different anterior pituitary cell types and many anterior pituitary cells respond to more than one hypothalamic-releasing hormone. These shared features have however rarely been addressed in human studies, while the interplay between hormones might be as important, or more important, as each separate hormonal axis. For example, with ageing or after menopause, levels of several hormones change concomitantly. While this might reflect separate mechanisms, these hormonal changes could also be synchronised with each other and their concerted impact might be larger than the sum of their individual impact on the ageing phenotype. Also, in other systems and organs of the body, interplay, interaction, and networks are highly important for maintenance of homeostasis and proper functioning of the human body.

Little is known about the interrelationships of hormones from different hypothalamic-pituitary-target gland axes, especially over time, since patients, or healthy individuals, are rarely sampled multiple times during the day. Some studies did collect hormonal time series data and investigated associations between pituitary hormones and/or hormones from an endocrine target gland. For example, in patients with Cushing syndrome, who display excessive production of cortisol, pulsatile TSH secretion is suppressed and irregular [3]. TSH secretion is also decreased in patients with acromegaly who display excessive production of GH [4]. However, these studies were performed in patients, so the observed relationships could be influenced by other aspects of their illness and not only by the altered hormone secretion. Few studies have been performed in healthy individuals. For example, Vis *et al.* assessed hormonal relationships in 18 obese women and found among others relationships between ACTH and cortisol, TSH and GH, TSH and cortisol, and between TSH and ACTH [5]. Furthermore, glucocorticosteroid administration

directly supressed pulsatile TSH secretion in healthy men [6] and a positive cross-correlation between GH and cortisol was found in older men and women [7].

In the present study, we aimed to determine the interrelationships between GH, TSH, ACTH, and cortisol in healthy older men and women. To this end, we analysed 24-h hormone concentration series assessed at intervals of 10 min from 38 healthy older participants from the Leiden Longevity Study [8]. Moreover, we examined whether interrelationships between hormones differ between men and women or between offspring of long-lived families and their partners. Furthermore, differences between interrelationships during the lights-on and -off periods were determined. We performed cross-correlation analyses to assess the relative strength between two 24-h hormone concentration series for all possible time shifts and cross-approximate entropy (ApEn) to assess pattern synchronicity between the different 24-h hormone concentration series.

METHODS

Study participants

In the Switchbox Leiden Study, we collected 24-h blood samples from 38 healthy older (range 52-76 years) individuals comprising 20 men and 18 women between June 2012 and July 2013 [9]. Participants were recruited from the Leiden Longevity Study, which is a family-based study consisting of 421 families with at least two long-lived siblings (men ≥ 89 years and women ≥ 91 years) together with their offspring and the offspring's partners without any selection on health or demographics [10]. The Switchbox Leiden Study comprised of 20 offspring of long-lived families, including 10 men and 10 women, and 18 partners of the offspring as a control group, including 10 men and 8 women. Participants had a stable body mass index (BMI) between 20-34 kg/m^2 and although not formally asked, based on the age range, the majority of women was most likely postmenopausal. Exclusion criteria were having a fasting plasma glucose above 7 mmol/L, having chronic renal, hepatic or endocrine disease, or using medication known to influence lipolysis, thyroid function, glucose metabolism, GH or IGF-1 secretion and/or any other hormonal axis. Hence, none of the participants were using estrogen-containing compounds. Participants were excluded if they had a recent trans meridian flight or when they recently performed shift work. To be able to safely perform the 24-h blood sampling, other exclusion criteria were difficulties to insert and maintain an intravenous catheter, anaemia (haemoglobin below 7.1 mmol/L), and blood donation within the last two months. The Switchbox Leiden Study protocol was approved by the Medical Ethical

Committee of the Leiden University Medical Centre and performed according to the Helsinki declaration. All participants gave written informed consent for participation.

Study protocol

Participants were admitted to the Research Centre at 08:00 h where a catheter was placed in a vein of the forearm of the nondominant hand. Blood sampling started around 09:00 h and every 10 min, 2 ml of blood was collected in a serum tube and 1.2 ml in an EDTA tube [8]. The participants received standardized feeding consisting of 600 kcal Nutridrink (Nutricia Advanced Medical Nutrition Zoetermeer, The Netherlands) at three fixed times during the day (between 09:00 and 10:00 h, 12:00 and 13:00 h, and 18:00 and 19:00 h). Lights were switched off between 23:00 and 08:00 h to allow the participants to sleep and except for lavatory use, no physical activity was allowed during the study period. All participants were sampled in the same research room. Anthropometric measurements, comprising weight, height, waist circumference, fat mass, and lean body mass were performed in the Research Centre using a scale, measuring tape, and Bioelectrical Impedance Analysis at a fixed frequency of 50 kHz (Bodystat® 1500 Ltd., Isle of Man, British Isles). Body mass index (BMI) was calculated as weight (in kilograms) divided by the square of height (in meters). Data on habitual bedtime and getting up time during the past month were obtained using the Pittsburgh Sleep Quality Index questionnaire [11].

Biochemical assays

All laboratory measurements were performed with fully automated equipment and diagnostics from Roche Diagnostics (Almere, The Netherlands) and Siemens Healthcare diagnostics (The Hague, The Netherlands) at the Department of Clinical Chemistry and Laboratory Medicine of the Leiden University Medical Center in The Netherlands. Full details on the procedures of the hormone assays have been described previously [9, 12, 13]. Levels of GH, TSH, cortisol, and ACTH were all measured in blood samples collected every 10 min from all 38 participants. Human growth hormone with a molecular mass of 22 kDa was measured in serum samples using Siemens reagents and an IMMULITE® 2000 Xpi Immunoassay system (Siemens Healthcare diagnostics). TSH and cortisol were measured in serum samples by ECLIA (ElectroChemoLuminescence ImmunoAssay) using cobas reagents and a Roche Modular E170 Immunoanalyser. ACTH was measured in EDTA samples using Siemens reagents and an IMMULITE® 2000 Xpi Immunoassay system. The data was checked for obvious outliers by visual inspection of a graphical display of individual hormone profiles from all 38 participants [14]. This was performed by four reviewers with expert knowledge in endocrinology. After reviewing the data

individually, a consensus meeting was held to reach agreement on data points which only one or two reviewers had marked as an outlier. In total for 28 out of 38 participants, 1.1 (SD = 1.8) data points per hormonal concentration series were on average detected as outliers and excluded from the dataset. Glucose and insulin were measured in a fasting serum sample withdrawn around 08:30 h at the second day of the 24-h blood sampling. Glucose was measured using Roche Hitachi Modular P800 and insulin was measured using IMMULITE® 2000 Xpi Immunoassay.

Cross-correlation

Cross-correlation assesses the relative strength between two 24-h hormone concentration series for all possible time shifts, by calculating linear Pearson's correlation coefficients as explained in more detail elsewhere [15, 16]. For example, hormone concentrations in time series A are compared pairwise with those of series B measured simultaneously (zero lag) or measured earlier or later (with a time lag). The unit of one lag time is the interval between two sampling points, so a lag time of 1 means that there is a delay of 10 min between two time series. Cross-correlation analyses were performed using the ccf function in the software program R, version 3.4.3 (The R Foundation for Statistical Computing, Vienna, Austria). The range of tested lag times depends on the number of data points in one time series; the range is lag -18 to 18 (360 min in total) for 144 data points. A correlation is considered significant when the absolute value is greater than $2/(\sqrt{n - |k|})$, where n is the number of data points in one time series and k is the maximal possible lag [17]. For a time series of 144 data points and a maximal lag of 18, the significance level is 0.18. Cross-correlation analyses were also performed after stratifying the 24-h data for lights-on period, which is the data from time point 09:00 h up to and including 22:50 h, and lights-off period (23:00 to 08:00 h). For these sub analyses, the lag range and the significance level changed accordingly to a lag range of -16 to 16 (320 min) and significance level of 0.24 for the lights-on period, and a lag range of -14 to 14 (280 min) and significance level of 0.31 for the lights-off period.

Cross-ApEn

Bivariate cross-approximate entropy (Cross-ApEn) quantifies joint pattern synchrony between two simultaneously measured time series, with lower cross-ApEn values signifying greater synchrony [18, 19]. Synchrony refers to pattern similarity, so to what extent sub patterns of window length m in time series A appear in time series B with a certain margin (r). Cross-ApEn was calculated for $m = 1$ and $r = 0.2$ (20% of the SD of the individual subject's hormone time series) with standardized data using the Matlab software program (Mathworks, Inc., Natick, MA, USA). Subsequently, jackknifing was

performed, which is a rigorous and objective leave-one-out cross-validation test that gives less bias in smaller samples than regular cross-ApEn, and it is more applicable for hormone data. It is important to note that a cross-ApEn of hormones A-B is different from a cross-ApEn of hormones B-A, since A is leading in the first case and following in the second. Cross-ApEn analyses were also performed after stratifying the 24h data for lights-on period, which is the data from time point 09:00 h up to and including 22:50 h, and lights-off period (23:00 to 08:00 h). Since cross-ApEn analyses cannot deal with missing data, missing data points were linearly interpolated.

Statistical analysis

Characteristics of the study participants were calculated using descriptive statistics. Normally distributed variables were presented as mean with standard deviation and differences between men and women and between offspring and partners were assessed by independent-samples t-tests. Not normally distributed variables were presented as median with interquartile ranges, using the nonparametric independent-samples Mann-Whitney U test to assess differences between subgroups. All statistical analyses were performed using SPSS for Windows, version 23 (SPSS, Chicago, IL, USA). Tukey box plots were made using GraphPad Prism version 7 (GraphPad, San Diego, CA, USA).

RESULTS

Characteristics of study participants

Characteristics of study participants are presented in Table 1 for all participants and stratified for sex and offspring-partner status. The number of men is equal in offspring and partner groups. Men and women were also similar in their offspring-partner distribution. Participants had a mean (SD) age of 65.1 (5.1) years, which was similar for men and women and for offspring and partners. The observed median (IQR) BMI of 24.8 (22.2 – 28.0) kg/m^2 is normal for this age category and was similar for all subgroups. Fasting glucose and insulin levels were for all participants within the reference range of our laboratory, with similar levels between groups. As expected, men and women differed in measures of body composition, with men being taller, having less fat mass, more lean body mass, and larger waist circumference than women. Groups of offspring and partners were similar in body composition. Participants were normal nocturnal sleepers in the month prior to the study day with a median (IQR) habitual bedtime of 23:30 (23:00 – 00:00) h and getting up time of 08:00 (07:30 – 08:15) h, which is similar to the time schedule of the study protocol during the 24-h blood sampling. Habitual bedtimes and getting up times were similar for men and women and for offspring and partners.

Table 1. Characteristics of study participants, for all subjects and stratified for sex and family history

		Stratified for sex			Stratified for family history		
	All subjects *n* = 38	**Men *n* = 20**	**Women *n* = 18**	***P* value**	**Offspring *n* = 20**	**Partners *n* = 18**	***P* value**
Male, *N* (%)	20 (52.6)	NA	NA	NA	10 (50)	10 (55.6)	0.76
Offspring of long-lived family, *N* (%)	20 (52.6)	10 (50)	10 (55.6)	0.76	NA	NA	NA
Age (years)[a]	65.1 (5.1)	65.6 (5.3)	64.6 (5.0)	0.56	65.6 (5.4)	64.6 (4.9)	0.52
BMI (kg/m^2)[b]	24.8 (22.2 – 28.0)	25.2 (23.3 – 27.4)	23.1 (21.6 – 29.9)	0.21	24.8 (22.3 – 29.3)	25.1 (22.1 – 27.7)	0.96
Height (cm)[b]	175 (165 – 181)	178 (175 – 182)	165 (162 – 168)	<0.001	175 (164 – 180)	175 (167 – 182)	0.58
Fat mass (kg)[b]	20.5 (18.5 – 27.0)	19.1 (18.0 – 24.1)	23.5 (19.7 – 34.7)	0.02	21.9 (18.7 – 27.5)	20.4 (18.4 – 29.1)	0.78
Lean body mass (kg)[b]	53.3 (41.5 – 62.2)	60.5 (57.6 – 66.0)	41.5 (37.4 – 44.8)	<0.001	52.4 (41.8 – 62.8)	54.3 (40.4 – 63.0)	0.73
Waist circumference (cm)[b]	94 (82 – 100)	97 (92 – 106)	82 (80 – 95)	0.001	92 (82 – 101)	94 (83 – 98)	0.96
Fasting glucose [mmol/L][a]	4.9 (0.6)	4.9 (0.7)	4.9 (0.5)	0.98	4.9 (0.7)	4.8 (0.4)	0.51
Fasting insulin [mU/L]	5.7 (3.7 – 7.9)	6.2 (3.4 – 10.1)	5.1 (3.9 – 6.3)	0.44	4.5 (3.5 – 8.0)	5.9 (3.8 – 7.8)	0.78
Habitual bedtime (h)	23:30 (23:00 – 00:00)	23:30 (23:00 – 23:45)	23:30 (23:00 – 23:45)	0.68	23:30 (23:00 – 00:00)	23:30 (23:00 – 23:30)	0.92
Habitual getting up time (h)	08:00 (07:30 – 08:15)	07:45 (07:00 – 08:15)	08:00 (07:30 – 08:15)	0.23	08:00 (07:30 – 08:30)	07:45 (07:00 – 08:15)	0.35

Unless indicated otherwise, data are presented as median with interquartile ranges. [a]Data are presented as mean with standard deviation. [b]Data were not available for one male partner. [c]Data were not available for one female offspring. NA = not applicable.

Cross-correlations of GH, TSH, ACTH, and cortisol

Figure 1 presents the cross-correlations between TSH and GH (a), cortisol and TSH (b), ACTH and cortisol (c), cortisol and GH (d), ACTH and GH (e), and ACTH and TSH (f) in all 38 participants. For TSH and GH, the maximal correlation was found at lag time 0 with a mean (95% CI) correlation coefficient of 0.29 (0.22 – 0.37). All cross-correlations between lag time -90 and 80 were positive. The strongest correlation between cortisol and TSH was found at lag time 170 with a mean (95% CI) correlation coefficient of -0.30 (-0.36 – -0.25). Also between lag times 90 and 180, cortisol and TSH were significantly negatively correlated, indicating that cortisol concentrations are negatively followed by TSH with a delay of 90 to 180 min. For ACTH and cortisol, the mean (95% CI) maximal correlation coefficient was 0.78 (0.74 – 0.81) at lag time 10, indicating that cortisol concentrations follow ACTH concentrations with a delay of 10 min. No significant cross-correlations between cortisol and GH, nor between ACTH and GH, were found. For ACTH and TSH, a weak maximal cross-correlation was observed at lag time 180 with a mean (95% CI) correlation coefficient of -0.19 (-0.24 – -0.15), which indicated that ACTH concentrations are negatively followed by TSH concentrations after 180 min.

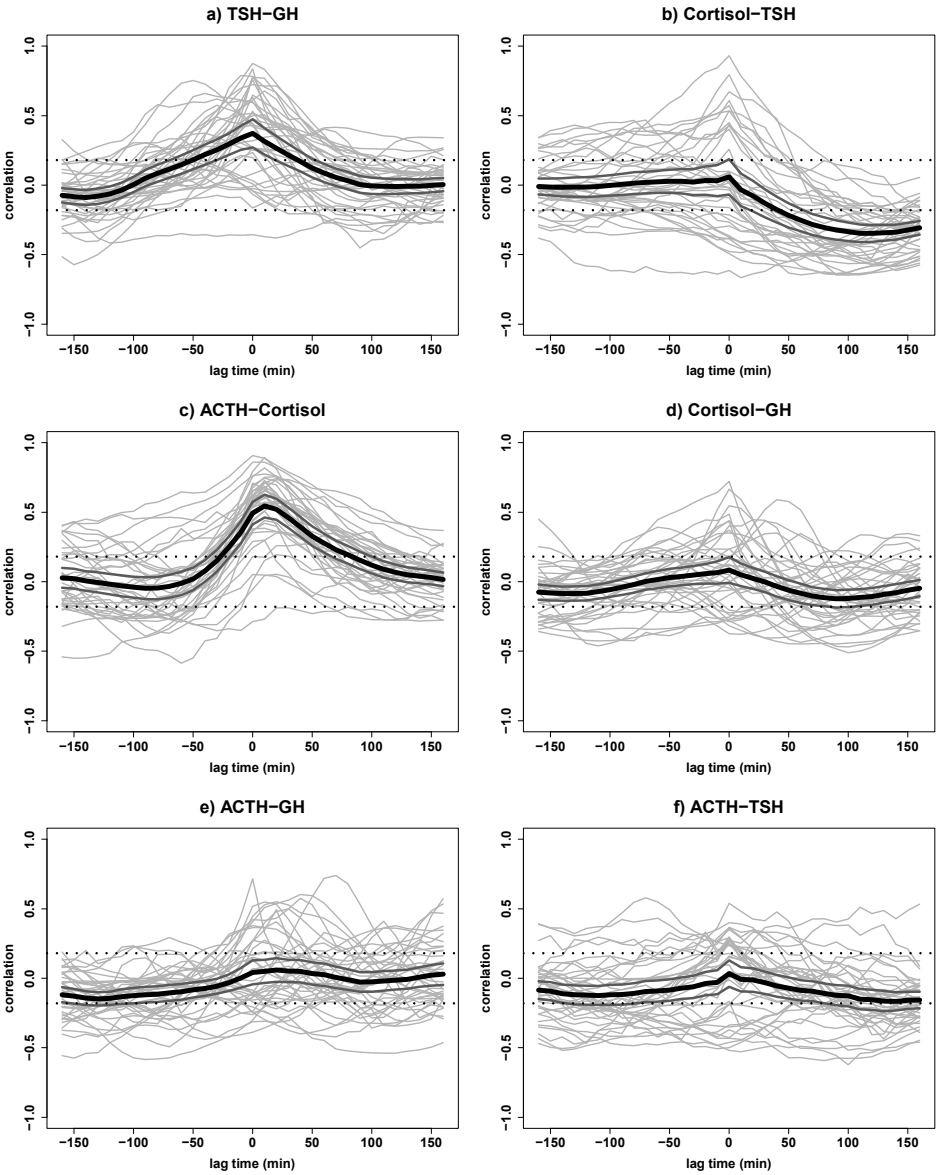
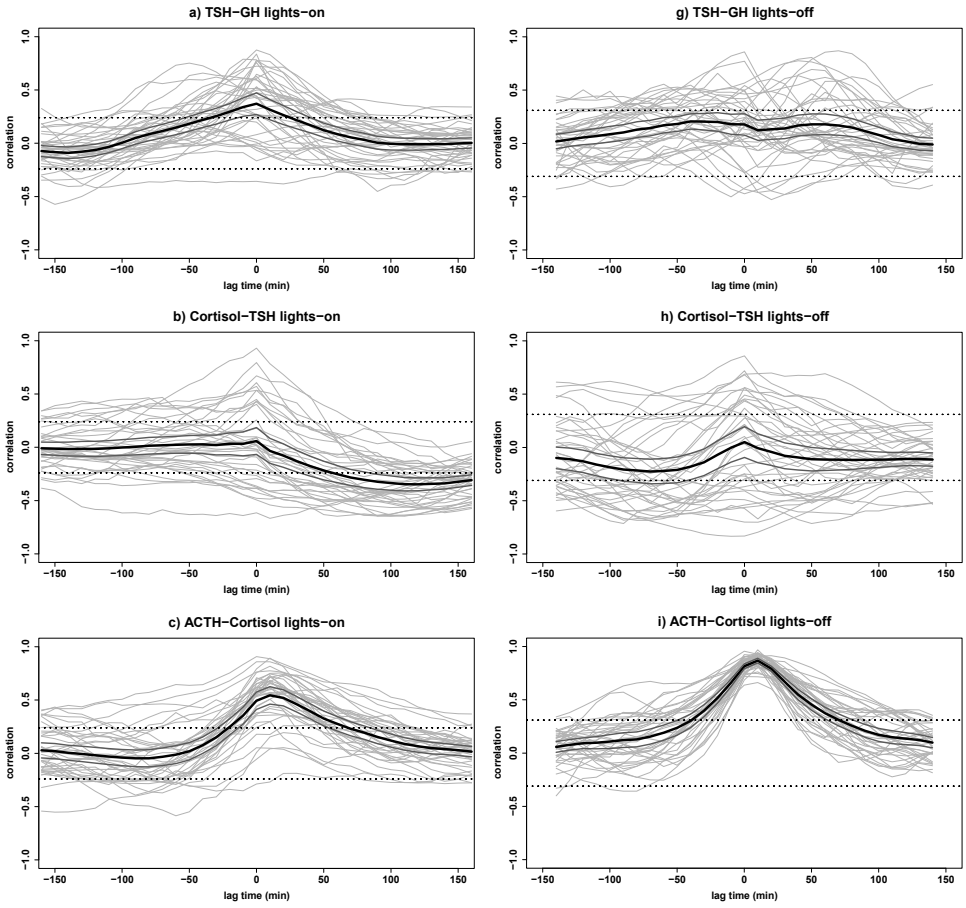


Figure 1. Cross-correlations of GH, TSH, ACTH, and cortisol.

Cross-correlations of a) TSH and GH, b) cortisol and TSH, c) ACTH and cortisol, d) cortisol and GH, e) ACTH and GH, and f) ACTH and TSH in all 38 participants. Cross-correlation assesses the relative strength between two hormone time series for all possible time shifts. The graph displays the correlation (y-axis) at a lag time in minutes (x-axis) with each grey line corresponding with one participant. The black line indicates the mean correlation for all participants and the two dark grey lines indicate the 95% confidence interval. The significance level is indicated by two straight dotted lines at correlations -0.18 and +0.18. Negative lag times represent a correlation in which hormone 2 is followed by hormone 1 and positive lag times represent a correlation in which hormone 1 is followed by hormone 2.

Cross-correlations stratified for lights-on and lights-off periods

Figure 2 presents the cross-correlations of GH, TSH, ACTH, and cortisol stratified for lights-on and lights-off periods. In line with the correlation found between TSH and GH concentrations over the complete 24-h period, we observed a strong positive correlation at lag time 0 (0.37 (0.18 – 0.35)) during the lights-on period. However, the correlation between TSH and GH disappeared in the lights-off period. Also for cortisol and TSH, we observed similar results during the lights-on period as during the complete 24-h period. A negative correlation at positives lag times was found during the lights-on period, but no significant correlation was found during the lights-off period. The cross-correlation between ACTH and cortisol is stronger during the lights-off period (0.87 (0.85 – 0.89)), than during the lights-on period (0.55 (0.39 – 0.53)). No significant cross-correlations between cortisol and GH, between ACTH and GH, and between ACTH and TSH concentrations, were found after stratifying the 24-h data for lights-on and -off periods.



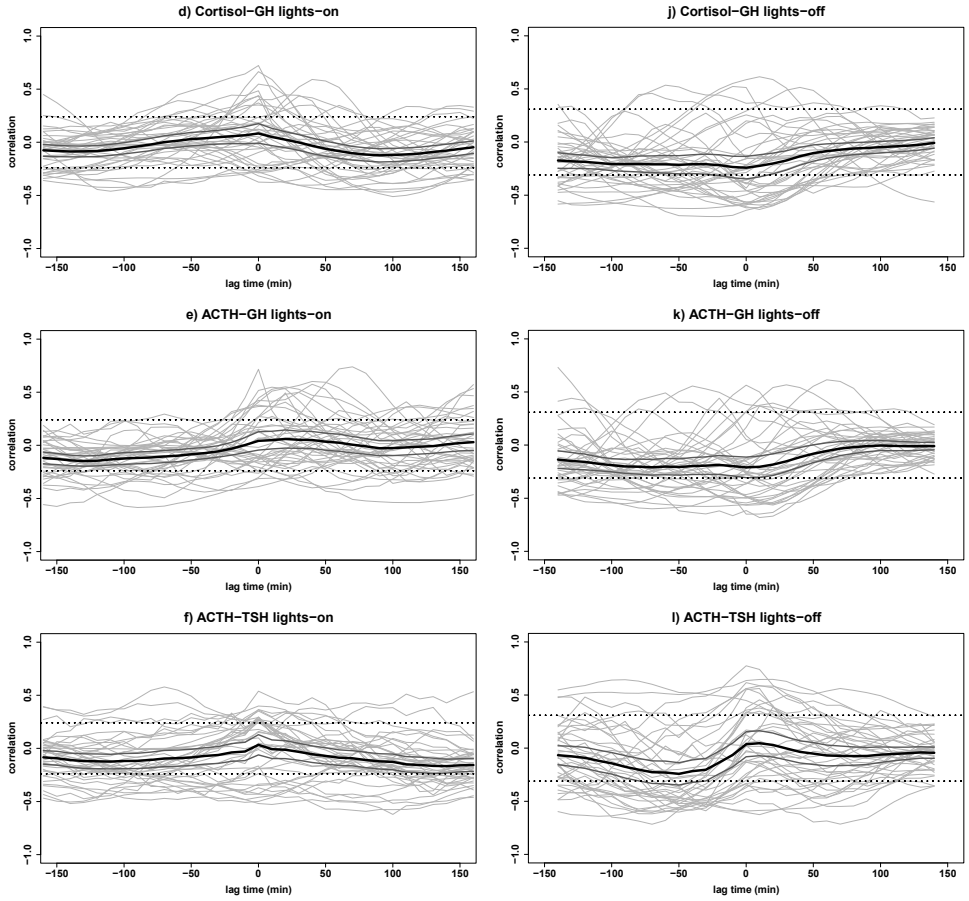


Figure 2. Cross-correlations of GH, TSH, ACTH, and cortisol stratified for lights-on and lights-off periods.

Cross-correlations of hormone combinations of GH, TSH, ACTH, and cortisol in all 38 participants stratified for lights-on period (a-f) from 09:00-22:50 h and lights-off period (g-l) from 23:00-08:00 h. Cross-correlation assesses the relative strength between two hormone time series for all possible time shifts. The graph displays the correlation (y-axis) at a lag time in minutes (x-axis) with each grey line corresponding with one participant. The black line indicates the mean correlation for all participants and the two dark grey lines indicate the 95% confidence interval. The significance level is indicated by the two straight dotted lines at correlations -0.24 and +0.24 for the lights-on period and at -0.31 and +0.31 for the lights-off period. Negative lag times represent a correlation in which hormone 2 is followed by hormone 1 and positive lag times represent a correlation in which hormone 1 is followed by hormone 2.

Cross-correlations stratified for men and women

Cross-correlation results of GH, TSH, ACTH, and cortisol were stratified for men and women. In Figure 3, a graphical summary of main findings from cross-correlation analysis are displayed for all participants (a) and for men (b) and women (c) separately. For TSH and GH, the maximal correlation in women was found at lag time 0 with a mean (95% CI) correlation coefficient of 0.27 (0.15 – 0.39). In men, the strongest cross-correlation (0.33 (0.24 – 0.43)) between GH and TSH was found at lag time -40 indicating that TSH concentrations are following GH concentrations with a delay of 40 min. However, also in men there were positive correlations at all lag times between -100 and 120 min. The strongest correlation between cortisol and TSH in men (-0.35 (-0.42 – -0.28)) was found at lag time 170, but in women, the strongest cross-correlation (0.32 (0.20 – 0.44)) was found at lag time 0, indicating that cortisol and TSH concentrations were strongly positively correlated without a delay. However, also in women we observed a weak but significant negative correlation at lag times 120 until 180 min. For ACTH and cortisol, similar results were observed in men (0.78 (0.73 – 0.83)) and women (0.78 (0.73 – 0.82)). No significant cross-correlations between cortisol and GH, and between ACTH and GH, were found after stratifying for men and women. In men, a weak mean correlation coefficient of -0.21 (-0.27 – -0.15)) at lag time 180 min was found between ACTH and TSH concentrations. In contrast, a weak positive correlation coefficient of 0.22 (0.11 – 0.33) was found at lag time 0 in women.

Cross-correlations stratified for offspring and partners

When cross-correlation results were stratified for offspring and partners, similar results were observed in offspring (0.32 (0.21 – 0.44)) and partners (0.26 (0.16 – 0.35)) for the cross-correlation of TSH and GH concentrations (data not shown). Also for cortisol and TSH, similar results were observed in offspring (-0.30 (-0.36 – -0.23)) and partners (-0.31 (-0.40 – -0.22)). The strongest cross-correlation coefficient for ACTH and cortisol concentrations in offspring was 0.75 (0.70 – 0.81), which was similar to their partners (0.80 (0.76 – 0.85)). No significant cross-correlations between cortisol and GH, and between ACTH and GH, were found after stratifying for offspring and partners. In partners, a mean negative correlation coefficient of -0.22 (-0.29 – -0.14) was found between ACTH and TSH concentrations. In contrast, no significant correlation between ACTH and TSH was observed in the offspring.

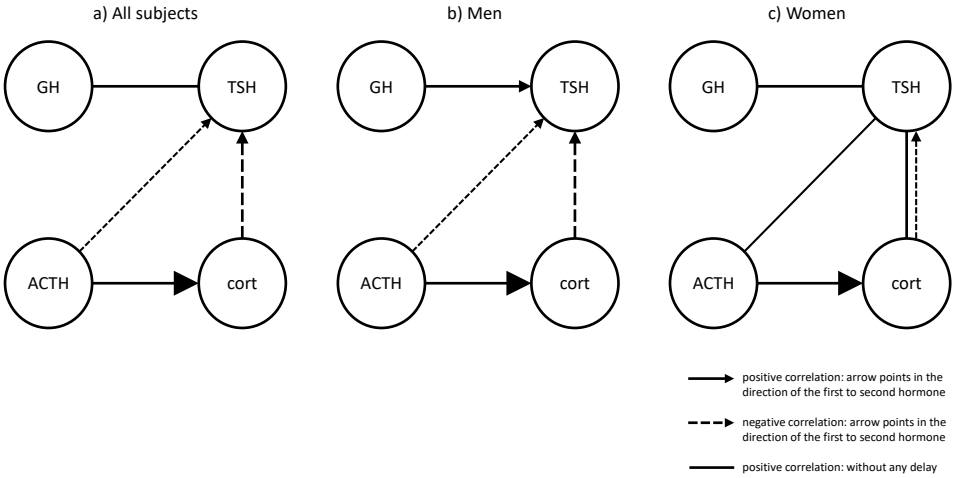


Figure 3. Summary of cross correlations in a) all subjects, b) men, and c) women.
A graphical summary of cross-correlation analyses in a) all 38 participants, b) 20 male participants, and c) 18 female participants. Solid lines represent positive correlations between hormones which is strongest at lag time 0, so without a delay. Solid arrows represent positive correlations between hormones which is strongest at a certain lag time, with the arrow directed towards the hormone which is following the leading hormone. Dotted arrows represent negative correlations between hormones which is strongest at a certain lag time, with the arrow directed towards the hormone which is (negatively) following the leading hormone. The weight of the line/arrow represents the strength of the correlation.

Cross-ApEn of GH, TSH, ACTH, and cortisol

Figure 4 presents box plots of cross-approximate entropy (cross-ApEn) results for hormone combinations of GH, TSH, ACTH, and cortisol. Values of cross-ApEn ranged from 0.5 to 2.3 and mean values ranged from 0.9 to 1.4 in all participants, with lower cross-ApEn values signifying greater joint pattern synchrony between two hormone concentration time series. The cross-ApEn between GH and cortisol was the lowest of all hormone combinations with a mean (95% CI) of 0.9 (0.8 – 1.0). Also the cross-ApEn values of GH-TSH, GH-ACTH, and cortisol-GH were lower than those of other hormone combinations. After stratifying for lights-on and lights-off periods, cross-ApEn values were significantly lower during the lights-on period compared with the lights-off period for the following hormone combinations: cortisol-TSH, GH-TSH, and GH-cortisol (data not shown). For cortisol-TSH, a mean (SE) difference of -0.17 (0.07) with a *P* value of 0.03 was found between lights-on and lights-off periods. The mean (SE) difference in cross-ApEn of GH-TSH was -0.21 (0.08) with $P = 0.01$ and for GH-cortisol, the mean (SE) difference was -0.15 (0.07) with $P = 0.04$. For the hormone combinations ACTH-GH, ACTH-cortisol, ACTH-TSH,

and TSH-ACTH, cross-ApEn values were lower during the lights-off period compared with the lights-on period where lower cross-ApEn signifies stronger synchronicity. For ACTH-GH, the mean (SE) difference in cross-ApEn between lights-on and lights-off periods was 0.24 (0.06) with a *P* value < 0.001. The difference in cross-ApEn between lights-on and lights-off periods was greatest for ACTH-cortisol with a mean (SE) difference of 0.27 (0.07) and a significance of *P* < 0.001. A mean (SE) difference of 0.15 (0.07) (P=0.03) for ACTH-TSH cross-ApEn values between lights-on and lights-off periods was found. Also the cross-ApEn of TSH-ACTH was lower during the lights-off period compared with the lights-on period with a mean (SE) difference of 0.17 (0.07) and *P* = 0.02. No significant differences between men and women were found, but in general men tended to have lower cross-ApEn values than women (data not shown). Also between offspring and partners no significant differences were observed (data not shown).

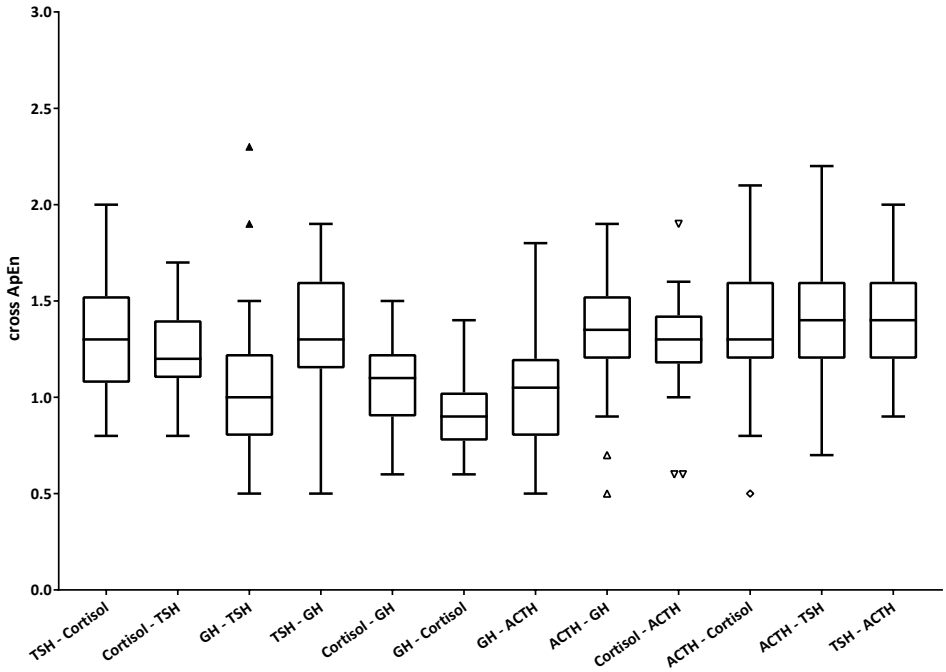


Figure 4. Cross ApEn for GH, TSH, ACTH, and cortisol.

Tukey box plots of the cross approximate entropy results of combinations of the hormones GH, TSH, ACTH, and cortisol in all 38 participants. Lower cross ApEn values signify greater synchrony between two hormone time series.

DISCUSSION

In this study, we aimed to determine the interrelationships between serum concentrations of GH, TSH, ACTH, and cortisol in healthy older individuals using 24-h hormone concentration series with intervals of 10 min. We confirmed that ACTH is positively correlated with cortisol, where cortisol follows ACTH with a delay of 10 min [20-22], and demonstrated that this correlation was stronger during night hours. Furthermore, we corroborate previous observations that cortisol and TSH concentrations are negatively cross-correlated in healthy older individuals [6, 23], where TSH follows cortisol concentrations with a delay of 170 min. Not earlier reported, a positive correlation between TSH and GH without any delay was found, which was more strongly during daytime. The cross-ApEn analyses showed that GH and cortisol exhibit synchronous serum concentration patterns. Several differences in cross-ApEn values were found between lights-on and -off periods, indicating that the pattern synchronicity between hormones is dependent on the time of the day. No major differences in cross-correlations were found between men and women, except for the positive correlation without any delay between cortisol and TSH concentrations, which was found in women but not in men. In general, men tended to have lower cross-ApEn values than women which was similar to other studies and which could indicate that postmenopausal women have reduced hormone pattern synchrony compared to men [9, 29]. No major differences in the interrelationships between hormones were found between offspring and partners.

Although cross-correlation and cross-ApEn analyses are complementary methods, the strong cross-correlation found between concurrent GH and TSH concentrations were strengthened by a relatively low cross-ApEn of GH-TSH pointing to strong synchronization between the two hormone concentration series. This strong interrelationship can probably not be explained by circadian synchronicity, since GH is mostly influenced by sleep and is less under circadian control [24]. Both TSH and GH play important roles in the regulation of energy metabolism, growth, and development, which could explain the presence of the interrelationship between TSH and GH concentrations. Additionally, we could speculate that this interrelationship between TSH and GH is established by the dopaminergic or somatostatinergic system, since these systems play regulatory roles in both the TSH and GH secretion [25]. Moreover, thyrotropin-releasing hormone (TRH) might stimulate, besides TSH, the secretion of GH, which was observed in a culture of rat cells [26]. During the embryonic development of the anterior pituitary, specific genes direct the cells toward a particular fate. For lactotrophs, somatotrophs, and thyrotrophs, the same genes are involved in their development until the final differentiation. This means that lactotrophs, somatotrophs, and thyrotrophs largely share the same

developmental cascade. Therefore, one might expect stronger correlations between TSH, GH, and prolactin than for example with ACTH, LH, or FSH. This might explain the strong correlation between GH and TSH.

The strong negative cross-correlation between cortisol and TSH indicates that cortisol concentrations are negatively followed by TSH with a delay of 90 to 180 min. Literature shows that glucocorticoids indeed suppress TSH secretion [6, 23, 27, 28] and it is believed that glucocorticoids exert a suprapituitary action. For both GH-TSH and cortisol-TSH, the cross-correlation as well as the pattern synchronicity were stronger during daytime than during night-time. One explanation why some of the significant cross-correlations disappeared after stratifying for lights-off periods could be that there are less data points during the lights-off period. This dilutes any effects and increases the threshold value for significance. Interestingly, we observed an even stronger correlation between ACTH and cortisol during the lights-off period, which potentially could be explained by the fact that the ACTH-cortisol system is maximally active during night-time. Similarly, cross-ApEn values of ACTH and cortisol were lower, indicating higher synchrony, during the night in this study, but also in healthy young subjects [20].

There is no cut-off value for significance for cross-ApEn values, but when comparing hormone pairs with each other, we found that the combination of GH and cortisol had the greatest joint pattern synchrony of all hormone combinations with higher synchrony during daytime. Another study found similar results for GH-cortisol cross-ApEn in healthy older men and women [7, 29]. Also other studies have shown a link between cortisol and GH in human [30, 31]. Other cross-ApEn values of hormone combinations with GH were relatively low as well, including the GH-TSH and GH-ACTH combinations, which could indicate that GH is interlinked with many different hormonal axes. We did not find a significant cross-correlation between GH and cortisol, which demonstrates that cross-correlation and cross-ApEn analyses are complementary methods. Cross-correlation assesses the strength between paired time series for all possible time shifts, resulting in linear lag-specific correlations, while cross-ApEn quantifies joint pattern synchrony between paired time series, which is lag-independent [32]. These methods therefore reflect different aspects of interrelationships between hormones.

This is one of the first studies in which interrelationships between hormones from different hypothalamic-pituitary-target gland axes over 24 h in healthy older subjects have been investigated by cross-correlation and cross-ApEn analyses. This innovative approach is a strength of the study although it makes it exploratory in nature as its novelty limits the

availability of similar studies. Cross-correlations between hormone concentrations are not evidence for a direct causal relationship between hormones. Furthermore, the potential day-to-day intra-subject variation remains unknown. Cross-ApEn is a validated tool to determine the joint pattern synchrony in a closed hormone system with known feedforward and feedback signals. However, cross-ApEn is rarely applied to combinations of hormones from different hormonal axes which makes it harder to interpret the biological meaning. Therefore, this study is more descriptive than conclusive. Nonetheless, it may promote the generation of new hypotheses on which future research can build.

It is assumed that offspring of long-lived families are biologically younger than their partners since among the key findings from the Leiden Longevity Study were the observations that the offspring had lower prevalence of myocardial infarctions, diabetes mellitus, hypertension, and metabolic syndrome compared to their partners [10, 33]. Therefore, we hypothesized that the offspring of long-lived families would have stronger hormonal interrelationships than controls. However, no major differences in the interrelationships between hormones were found between offspring and partners. This could indicate that this interplay between hormones is crucial for survival and if this interconnection would disappear, it would lead to illness. Participants in this study were selected based on their health status which resulted in a group of healthy older individuals and this could have influenced the results.

Hormones of the hypothalamic-pituitary-gonadal and the hypothalamic-pituitary-prolactin axes were not considered in this article. However, GH, TSH, ACTH, and cortisol might also interact with these hormones. Especially since lactotrophs, somatotrophs, and thyrotrophs largely share the same developmental cascade. Indeed, studies showed a positive association between the hypothalamic-pituitary-thyroid axis and prolactin; TRH regulates the synthesis and release of prolactin [34, 35], and Saini *et al.* found concurrent pulses of TSH and prolactin in young men [36]. Furthermore, prolactin was positively correlated with GH, TSH, and ACTH without any delay and with cortisol at a lag of 10 min in obese women [5]. Also the hypothalamic-pituitary-gonadal axis seems to be associated with other hormonal axes; long-term testosterone administration resulted in increased overnight GH secretion in healthy older men [37] and prolactin concentrations increased in response to oestrogen treatment in postmenopausal women [38].

CONCLUSION

This study demonstrates that interrelations between hormones from interlinked as well as different hypothalamic-pituitary-target gland axes are observed in (older) individuals. In particular, the correlations between cortisol and TSH concentrations, between TSH and GH concentrations, and the great joint pattern synchrony between GH and cortisol, are indications that distinct hormonal axes interact in healthy older individuals. No major differences were found between men and women, except for the positive correlations between cortisol and TSH concentrations found in women. Also no major differences between offspring of long-lived families and partners were found. The cross-correlation and pattern synchronicity between TSH and GH, and the pattern synchronicity between cortisol and TSH, were stronger during daytime than during night-time, but the cross-correlation and pattern synchronicity between ACTH and cortisol were stronger during night-time. More research is needed to determine the biological meaning and clinical consequences of these interrelationships between pituitary hormones.

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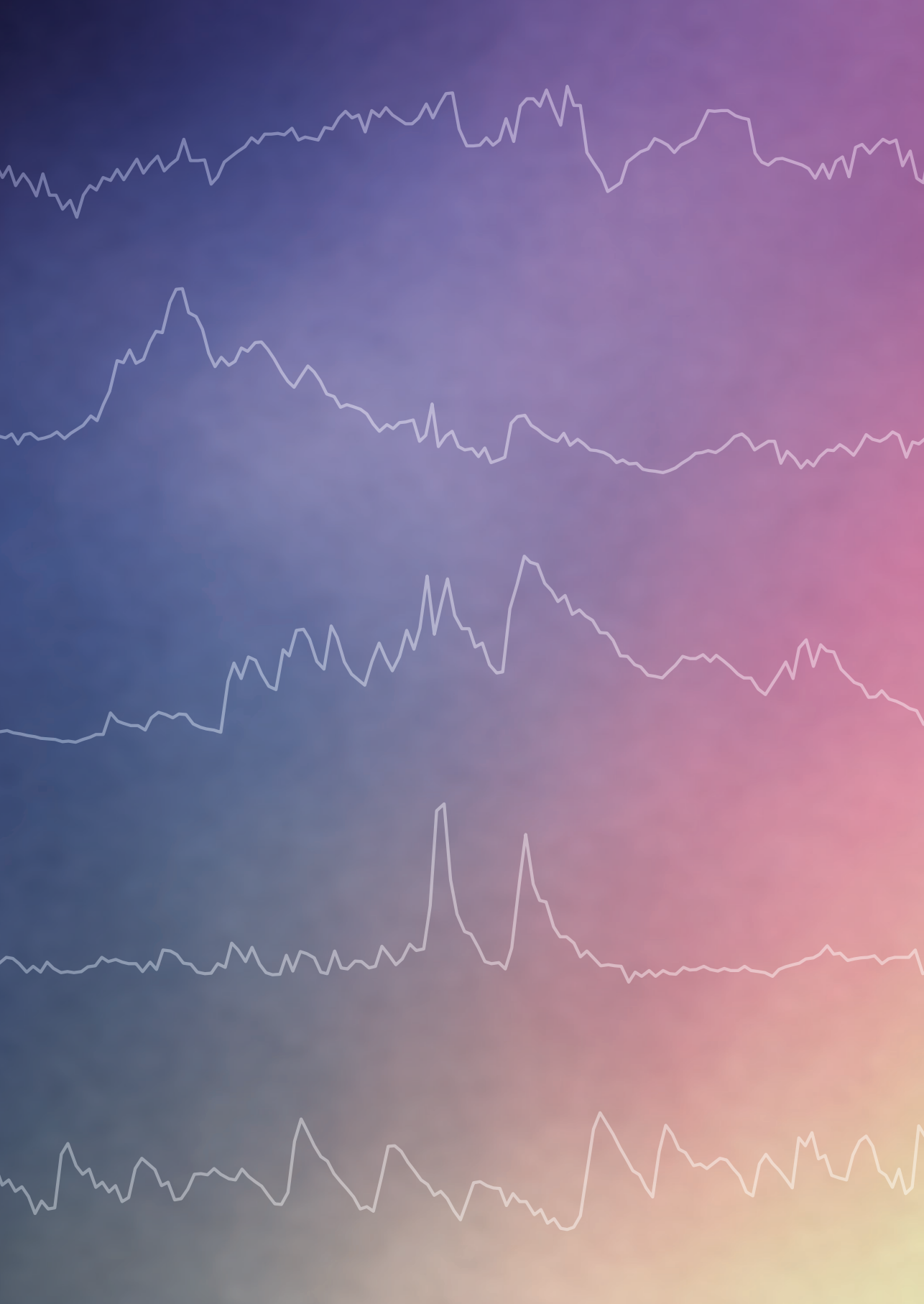

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The 24-hour serum profiles of bone markers in healthy older men and women

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ABSTRACT

The process of bone turnover displays variations over 24 h, with C-terminal cross-linked telopeptide of type 1 collagen (CTX) and osteocalcin exhibiting a nadir in the afternoon and a peak in the night. In contrast, N-terminal propeptide of type 1 procollagen (P1NP) did not display an apparent 24-h rhythm. Other emerging novel biomarkers of bone, sclerostin and Dickkopf-related protein 1 (DKK1), are markers of osteocyte activity with limited data available regarding their 24-h profiles. In this study, we aimed to extend available data on 24-h profiles of CTX, osteocalcin, and P1NP and to assess the 24-h profiles of sclerostin and DKK1 in healthy older men and women and to compare these between men and women. We measured these five bone markers in EDTA plasma collected every 4 h during 24 h in 37 healthy older men and women (range 52–76 years). Differences between time points were determined using repeated measures ANOVA and cosinor analyses were performed to determine circadian rhythmicity. The circadian rhythm of CTX was confirmed by the cosinor model, with women showing larger amplitude compared to men. Osteocalcin showed higher levels during night-time compared to daytime in both men and women. For P1NP levels we observed a small but significant increase in the night in men. Sclerostin and DKK1 did not show a circadian rhythm, but sclerostin levels differed between time points. Because of the large intraindividual variation, DKK1 as measured in this study cannot be considered a reliable marker for diagnostic or research purposes. In conclusion, when measuring CTX, osteocalcin, P1NP, or sclerostin either in clinical practice or in a research setting, one should consider the 24-h profiles of these bone markers.

INTRODUCTION

The process of bone turnover displays circadian variations, which are most pronounced for bone resorption as measured with C-terminal cross-linked telopeptide of type 1 collagen (CTX). The circadian rhythm of this key biomarker of bone resorption has been described extensively in literature. CTX is released into the blood circulation when bone collagen is broken down by cleavage of the cross-linked type I collagen by cathepsin K, which is expressed by osteoclasts [1, 2]. Serum CTX demonstrates a circadian rhythm with its nadir in the late afternoon and its peak in the second half of the night in healthy individuals [3-6]. The circadian rhythms of the two key biomarkers of bone formation, osteocalcin and N-terminal propeptide of type 1 procollagen (P1NP), have been investigated in several studies. Osteocalcin is produced by osteoblasts at sites of new bone formation. Most of the newly synthesized osteocalcin is incorporated into the bone matrix, while a small fraction is released into the circulation [7]. Its circadian rhythm has first been described in 1985 and similarly as CTX, serum osteocalcin has its nadir in the afternoon and its peak at night [8]. P1NP is enzymatically cleaved off from type 1 procollagen when bone is formed by osteoblasts [9]. Evidence regarding the 24-h rhythm of P1NP is yet inconclusive; two studies showed that P1NP does not exhibit a discernible 24-h rhythm while another study did identify a 24-h rhythm in P1NP with somewhat higher levels in the night [6, 10, 11].

Other emerging novel biomarkers of bone are markers of osteocyte activity, such as sclerostin and Dickkopf-related protein 1 (DKK1). Osteocytes are endocrine mechanosensory cells that, besides regulating phosphate and calcium homeostasis, control bone remodelling by regulating both osteoblasts and osteoclasts via cell-to-cell communication and secreted factors [12]. Sclerostin and DKK1 are negative regulators of bone formation that inhibit osteoblast activity via blocking the Wnt signalling pathway by antagonizing the Wnt/lipoprotein receptor-related protein 5 [13, 14]. Since bone formation and bone resorption demonstrate circadian rhythmicity and osteocytes regulate these processes, osteocyte activity will potentially also display a rhythm. Osteocytes regulate bone turnover via secreted regulatory proteins and therefore we hypothesized that these markers demonstrate a circadian rhythm similar to that of other bone markers. Only limited data are however available regarding the circadian rhythm of these emerging markers. No discernible 24-h rhythm was identified for sclerostin in men [6] while DKK1 levels have not yet been measured during 24 h.

Several factors, including age, sex, and postmenopausal status, have been investigated as potential modulators of the levels and circadian rhythm of bone turnover markers. Serum

levels of CTX in both men and women decreased with age until 40-50 years followed by a gradual increase in men and a sharp increase in women after menopause. CTX levels were lower in premenopausal women than in men, but higher in postmenopausal women [15-17]. In contrast to the serum levels, the circadian rhythm of serum CTX was not influenced by age, sex, or menopausal status [18]. For osteocalcin, levels decreased with age in both men and women, but increased slightly after age 65 for men and were increased after menopause in women. Osteocalcin levels are higher in young men compared to premenopausal women, but lower in older men compared to postmenopausal women [17, 19, 20]. Similar to CTX, the circadian rhythm of osteocalcin was not influenced by age, sex, or menopausal status [21-24]. P1NP levels decreased after 20 years of age in men but remained relatively stable afterwards. In women, levels of P1NP decreased until menopause and then started to increase. P1NP levels were lowest in premenopausal women, compared to men, and highest in postmenopausal women [15, 16]. No difference in the circadian rhythm of P1NP was found between younger and older healthy men [6]. Whether the circadian rhythm of P1NP is also independent of sex is not known yet. Sclerostin serum levels correlated positively with age in men, premenopausal, and postmenopausal women, with postmenopausal women having higher levels than premenopausal women [25-29]. Sclerostin levels were higher in men than in women [26, 28, 30]. The circadian rhythm of sclerostin has only been examined in men, so the difference in circadian rhythm between men and women is not known. Both in younger and older men, no circadian rhythm of sclerostin has been observed [6]. For DKK1, levels were higher in female than in male geriatric patients [30]. DKK1 levels were somewhat higher in older individuals than in younger individuals [31]. Since the circadian rhythm of DKK1 has not been investigated yet, no factors influencing the circadian rhythm have been described.

In this paper, we investigated for the first time the 24-h profiles of CTX, osteocalcin, P1NP, sclerostin, and DKK1 in the same participants, comprising 20 healthy older men and 17 healthy older women. Our objectives were to extend available data on 24-h profiles of CTX, osteocalcin, and P1NP and to assess the presence or absence of a circadian rhythm for sclerostin and DKK1 in healthy older men and women and to compare the 24-h profiles of these five bone markers between men and women.

METHODS

Study participants

In the Switchbox Leiden Study, we collected 24-h blood samples from 38 healthy older (range 52–76 years) individuals comprising 20 men and 18 women with the aim to assess 24-h fluctuations in circulating hormones and biomarkers, between June 2012 and July 2013. For the present analysis, one woman was excluded since she had self-reported osteoporosis and was using alendronic acid during the study. Consequently, 37 participants were included in the analyses. Participants of the Switchbox Leiden study were recruited from the family-based Leiden Longevity Study in which 421 long-lived families are included, comprising at least two nonagenarian siblings fulfilling the age criteria (men ≥ 89 years and women ≥ 91 years) without selection on health or demographics together with their offspring and the offspring's partners [32]. Participants of the Switchbox Leiden study had a stable body mass index (BMI) between 20 and 34 kg/m^2 and although not formally asked, based on the age range, the majority of women was most likely postmenopausal. Exclusion criteria were among others, having chronic renal, hepatic or endocrine disease, or using any hormone medication (including oral, nasal, and inhalation corticosteroids). Detailed information on in- and exclusion criteria can be found elsewhere [33]. The Switchbox Leiden Study was approved by the Medical Ethical Committee of the Leiden University Medical Centre and performed according to the Helsinki declaration. All participants gave written informed consent for participation.

Study protocol

Participants were admitted to the research centre during 24 h. A catheter was placed in a vein of the forearm of the non-dominant hand of the participant. Blood sampling started around 09:00 h and blood was collected every 10 min. For the current study we used EDTA plasma samples collected with four hour intervals (around 10:00 h, 14:00 h, 18:00 h, 22:00 h, 02:00 h, 06:00 h). The participants received standardized feeding at three fixed time intervals during the day (between 09:00 h–10:00 h, 12:00 h–13:00 h and 18:00 h–19:00 h), each consisting of 600 kcal Nutridrink (Nutricia Advanced Medical Nutrition Zoetermeer, The Netherlands). Participants were not allowed to sleep during the day, and except for lavatory use, no physical activity was allowed during the study period. Lights were switched off for approximately 9 h (circa between 23:00 h to 08:00 h). Full details on the 24-h blood sampling procedure have been described previously [34]. Anthropometric measurements, comprising BMI, height, fat mass, lean body mass, and waist circumference, were performed in the research centre using a scale, measuring tape, and a Bioelectrical Impedance Analysis meter at a fixed frequency of 50kHz (Bodystat® 1500

Ltd, Isle of Man, British Isles). Data on usual bed time and getting up time during the past month were obtained using the Pittsburgh Sleep Quality Index questionnaire [35].

Biochemical analysis

β-isomerized C-terminal cross-linked telopeptide of type 1 collagen (CTX), N-terminal propeptide of type 1 procollagen (P1NP), osteocalcin, sclerostin, and Dickkopf-related protein 1 (DKK1) were all measured in EDTA plasma samples collected every 4 h. Especially for DKK1 it is recommended to perform measurements in plasma instead of serum, as DKK1 is present in blood platelets. During the clotting process, platelets release their content, including DKK1, resulting in significantly higher DKK1 levels in serum compared to plasma [36]. Measurements of CTX, P1NP, and osteocalcin were performed with cobas® kits and the fully automated E170 module of Modular Analytics from Roche Diagnostics (Almere, The Netherlands) at the Department of Clinical Chemistry and Laboratory Medicine of the Leiden University Medical Centre in The Netherlands, which is accredited according to the National Coordination Committee for Quality Assurance for Health Care Laboratories in The Netherlands. CTX had an interassay coefficient of variation (CV) of 2.59% at 0.34 ng/mL and 2.16% at 0.782 ng/mL, osteocalcin of 0.91% at 20.1 µg/L and 1.33% at 93.0 µg/L, and P1NP had an interassay CV of 1.61% at 37.58 ng/mL and 1.83% at 210.77 ng/mL. Sclerostin was measured with a 96-well multi-array Human Sclerostin Assay from Meso Scale Diagnostics, with an interassay precision of 10%, as previously described [37]. DKK1 was measured by the Quantikine Human DKK-1 Immunoassay from R&D systems. The inter-assay CV of this kit is 7.6% at 243 pg/mL and 4.6% at 1371 pg/mL, and the interassay CV was 5.8% in our hands as previously described [38]. 25-hydroxyvitamin D, phosphate, calcium, and albumin were measured in a fasting morning serum sample, 25-hydroxyvitamin D using the E170 module of Modular Analytics and phosphate, calcium, and albumin using Modular P systems from Roche Diagnostics (Almere, The Netherlands). Calcium concentrations were corrected for albumin concentrations using the following formula: corrected calcium = measured calcium [mmol/L] + 0.02 * (42 – albumin [g/L]).

Statistical analysis

Characteristics of the study participants were calculated separately for men and women using descriptive statistics. Normally distributed variables were presented as mean with standard deviation and differences between men and women were assessed by independent-samples *t*-tests. Not normally distributed variables were presented as median with interquartile ranges, using the nonparametric independent-samples Mann-Whitney *U* test to assess differences between men and women. Mahalanobis distances

were calculated to check multivariate normality [39]. Two probabilities of Mahalanobis distances were below $P < 0.001$ for DKK1, so two participants (one man and one woman) were excluded for the DKK1 analyses. Differences between men and women in 24-h means of bone markers were calculated using the nonparametric independent-samples Mann-Whitney U test. We tested whether levels of bone markers were different between time points using repeated measures (RM) ANOVA, which is a model-independent method. RM ANOVA was performed with Time (10:00 vs 14:00 vs 18:00 vs 22:00 vs 02:00 vs 06:00) as within-subjects factor, Sex as between-subjects factor, and bone marker levels as dependent variables. Additionally, to take into account the effect of being offspring of long-lived family or partner, Status (offspring vs partner) was added as between-subjects factor and RM ANOVA analyses were repeated. Post-hoc independent-samples *t*-tests were performed to analyse differences between men and women per time point. When appropriate, paired-samples *t* tests were performed to calculate differences between mean values during day-time (time points 10:00, 14:00, 18:00 and 22:00) and mean values during night-time (time points 02:00 and 06:00). Furthermore, to determine whether a bone marker showed a circadian rhythm, cosinor analyses were performed using the software program R, version 3.4.3 (The R Foundation for Statistical Computing, Vienna, Austria). Cosinor analysis is a model-dependent method which fits a cosinor model to the raw data. First, the rhythm detection test, also called the zero-amplitude test, was performed to test the overall significance of the cosinor model. The cosinor model was fit with sex as covariate using the software package 'cosinor' developed by Michael Sachs and parameter tests were performed using the software package 'cosinor2' developed by Augustin Mutak [40, 41]. One of the circadian parameters calculated by the cosinor analysis is the midline estimating statistic of rhythm (MESOR), which is a circadian rhythm-adjusted mean based on the parameters of a cosine function fitted to the raw data. In addition, the amplitude is provided, which is the difference between the maximum and MESOR of the fitted curve. The acrophase represents the phase of the maximal value assumed by the curve [42]. Cosinor analyses were also performed with offspring-partner status as covariate to test whether being offspring of long-lived family or partner influenced the potential differences found between men and women. *P* values < 0.05 were considered as statistically significant. All statistical analyses, except for the cosinor analyses, were performed with SPSS for Windows, version 23 (SPSS, Chicago, IL, USA). Figure 1 and Supplementary Figure S1 were made using GraphPad Prism version 7 (GraphPad, San Diego, CA, USA) and Figure 2 was made using R, version 3.4.3, and Adobe Illustrator.

RESULTS

Characteristics of study participants

The characteristics of the study participants are presented separately for 20 men and 17 women in Table 1. Men and women were similar in their age, offspring-partner distribution, BMI, and vitamin D and calcium levels. Participants were normal nocturnal sleepers in the month prior to the study day, with similar usual bed time and getting up time for men and women. As expected, measures of body composition were different between men and women, with men being taller, having less fat mass, more lean body mass, and larger waist circumference. Phosphate levels were higher in women than in men.

Table 1. Characteristics of study participants stratified by sex

	Men *n* = 20	**Women *n* = 17**	***P* value**
Age (years)[a]	65.6 (5.3)	64.6 (5.2)	0.57
Offspring of long-lived family, *N* (%)	10 (50)	9 (52.9)	0.86
BMI (kg/m^2)[b]	25.2 (23.3 – 27.4)	23.2 (21.9 – 30.2)	0.32
Height (cm)[b]	178 (175 – 182)	165 (162 – 168)	<0.001
Fat mass (kg)[b]	19.1 (18.0 – 24.1)	23.5 (20.2 – 34.8)	0.01
Lean body mass (kg)[b]	60.5 (57.6 – 66.0)	41.5 (37.9 – 45.5)	<0.001
Waist circumference (cm)[b]	97 (92 – 106)	82 (81 – 95)	0.003
25-hydroxyvitamin D [nmol/L]	69.9 (54.8 – 86.1)	79.1 (61.4 – 101.1)	0.22
Phosphate [mmol/L][a,b]	0.98 (0.15)	1.12 (0.11)	0.003
Calcium [mmol/L][c]	2.46 (2.36 – 2.50)	2.46 (2.41 – 2.50)	0.64
Usual bed time (h)	23:30 (23:00 – 23:38)	23:30 (22:45 – 23:30)	0.48
Usual getting up time (h)	07:45 (07:00 – 08:15)	08:00 (07:30 – 08:15)	0.33

Unless indicated otherwise, data are presented as median with interquartile ranges. [a]Data are presented as mean with standard deviation. [b]Data were not available for one man. [c]Calcium concentration was corrected for albumin concentration.

24-h profiles of bone markers

Table 2 presents medians of 24-h means of CTX, osteocalcin, P1NP, sclerostin, and DKK1 with interquartile ranges for men and women separately. Figure 1 displays at time points 10:00, 14:00, 18:00, 22:00, 02:00, and 06:00 h the mean levels of bone markers stratified by sex. Individual 24-h profiles of all bone markers are plotted in Supplementary Figure S1. To investigate whether levels of bone markers differed over time, RM ANOVA was

performed with Time as within-subjects factor, Sex as between-subjects factor, and bone marker levels as dependent variable. When appropriate, post-hoc *t*-tests were performed to compare 24-h profiles between men and women. Furthermore, cosinor analyses were performed to determine a circadian rhythm, with Figure 2 displaying the cosinor model for CTX. Results are presented per bone marker in the following sections.

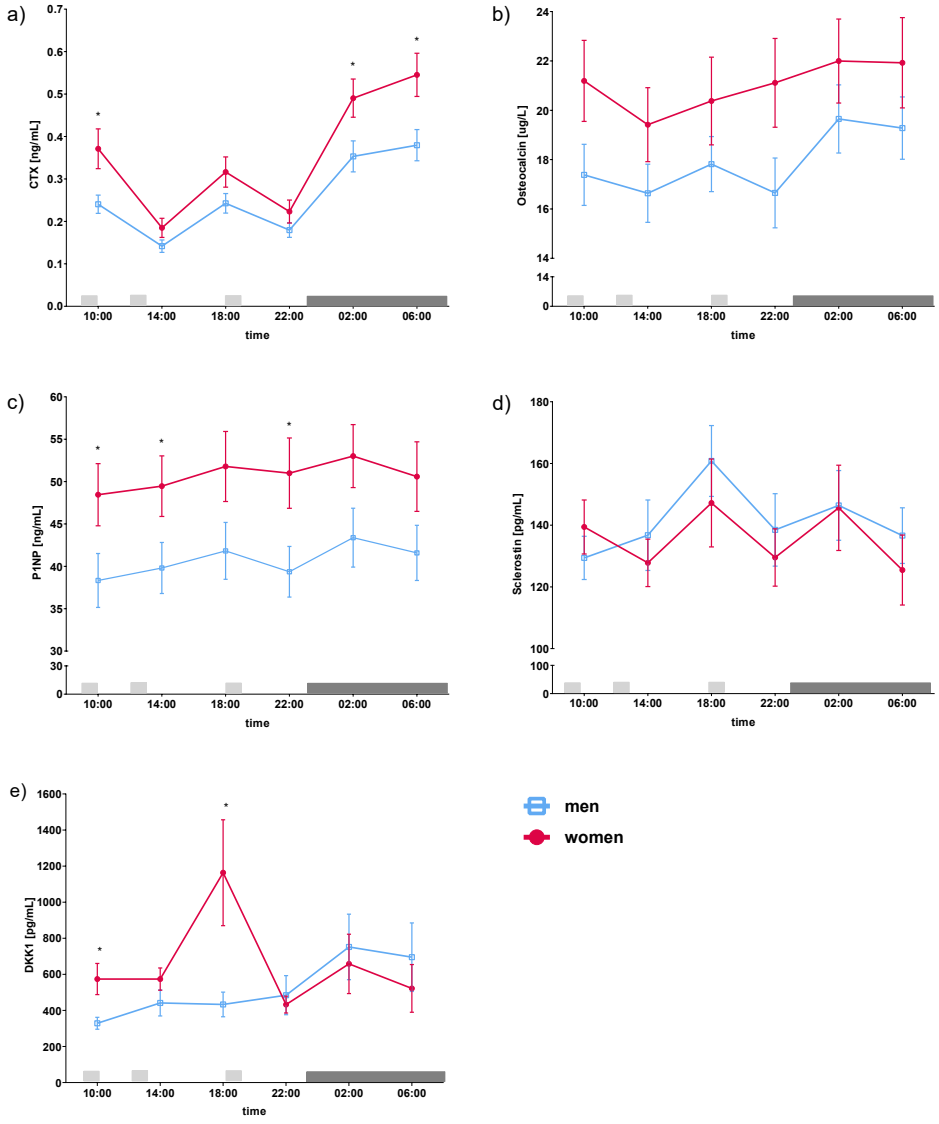

Figure 1. Bone markers over 24 hours.

The mean (SE) of a) C-terminal cross-linked telopeptide of type 1 collagen (CTX), b) osteocalcin, c) N-terminal propeptide of type 1 procollagen (P1NP), d) sclerostin, and e) Dickkopf-related protein 1

(DKK1) are presented every 4 hours starting at 10:00 during 24 hours for men (blue, light) and women (pink, dark). Stars (*) represent significant differences between men and women. Light bars represent meal times and dark bars represent the period when the lights were switched off.

Table 2. 24-h means of bone markers in study participants stratified by sex

	Men *n* = 20	**Women *n* = 17**	***P* value**
CTX [ng/mL]	0.23 (0.19 – 0.30)	0.32 (0.25 – 0.49)	0.02
Osteocalcin [µg/L]	16.6 (13.7 – 21.5)	22.0 (16.4 – 26.7)	0.045
P1NP [ng/mL]	40.1 (29.6 – 47.5)	52.2 (38.0 – 61.3)	0.11
Sclerostin [pg/mL]	134 (116 – 167)	129 (111 – 163)	0.11
DKK1 [pg/mL]	494 (347 – 753)	662 (455 – 855)	0.73

Data are presented as median 24-h means with interquartile ranges, analyzed by Independent Samples Mann-Whitney U test. Abbreviations: C-terminal cross-linked telopeptide of type 1 collagen (CTX), N-terminal propeptide of type 1 procollagen (P1NP), Dickkopf-related protein 1 (DKK1).

CTX

Women had significantly higher 24-h means of CTX levels compared to men (Table 2). Figure 1a displays mean levels of CTX for men and women separately at time points 10:00, 14:00, 18:00, 22:00, 02:00, and 06:00. By visual inspection, we observed that CTX exhibited in both men and women a decrease at 14:00 followed by an increase at 18:00 and a small decrease at 22:00. After 22:00, CTX increased with highest levels at 06:00. Mean CTX levels differed significantly between time points ($F(2.3,78.2) = 94.4$, $P < 0.001$) and the between-subjects factor Sex was also significant ($F(1,34) = 5.4$, $P = 0.03$). In addition, there was a Time by Sex interaction, ($F(2.3, 78.2) = 4.7$, $P = 0.01$), mainly showing higher mean CTX levels in the night in women than in men. Post-hoc *t*-tests indicated that women had significantly higher levels of CTX at 10:00 ($t_{df} = 2.7_{35}$, $P = 0.01$), 02:00 ($t_{df} = 2.4_{35}$, $P = 0.02$), and 06:00 ($t_{df} = 2.7_{35}$, $P = 0.01$) compared to men. Results were not materially different when Status (offspring vs partner) was added as a between-subjects factor in the analyses. The trajectory of CTX levels fitted a cosinor model over 24 h ($P < 0.001$), with a mesor estimate (95% confidence interval (CI)) of 0.26 (0.19 – 0.32) ng/mL in men ($P < 0.001$) and 0.35 (0.32 – 0.38) ng/mL in women ($P < 0.001$). Figure 2 displays the cosinor model fitted to CTX in men and women. The amplitude (95% CI) of CTX was estimated as 0.16 (0.12 – 0.20) ng/mL in women, which was significantly ($P = 0.04$) higher compared to men (0.10 (0.06 – 0.14) ng/mL). The acrophase estimate (95% CI) was 04:18 (02:46 – 05:50) in men ($P = 0.02$) and 04:54 (03:51 – 05:57) in women ($P = 0.03$), which was not significantly different between men and women ($P = 0.49$).

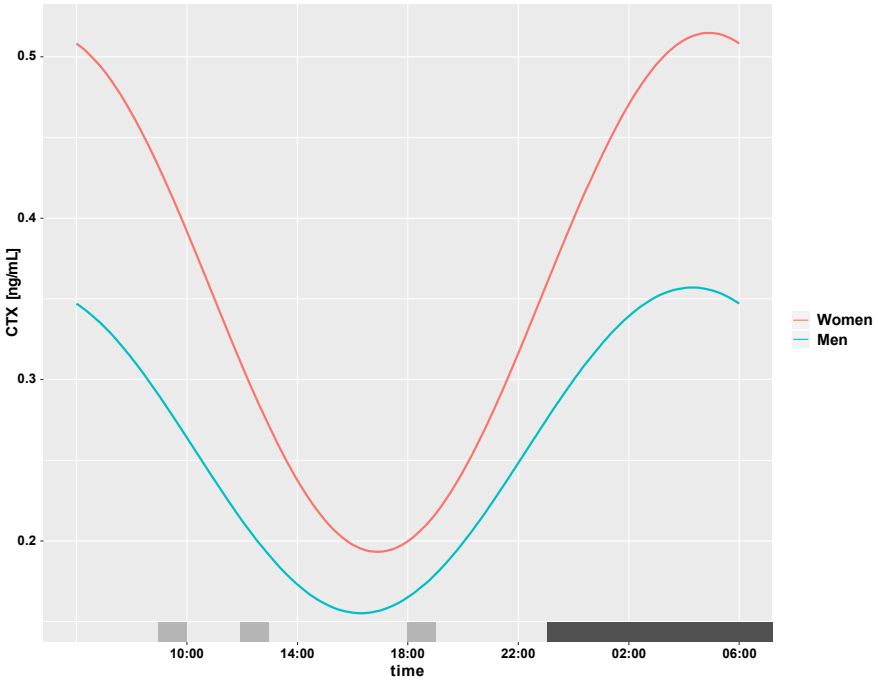

Figure 2. Cosinor model of CTX for study participants stratified by sex.

A cosinor model fitted to C-terminal cross-linked telopeptide of type 1 collagen (CTX) of 20 men (blue/lower line) and 17 women (red/upper line). Light bars represent meal times and the dark bar represents the period when the lights were switched off.

Osteocalcin

For osteocalcin, we observed higher 24-h means of osteocalcin levels in women than in men (Table 2). The 24-h profiles of osteocalcin are presented in Figure 1 with women showing a small decrease at 14:00 followed by a linear increase with its highest points at night (02:00 and 06:00). For men, osteocalcin levels were relatively constant during daytime and were increased in the night. Mean osteocalcin levels were significantly different between time points ($F(3.9,135.9) = 8.1$, $P < 0.001$). To test whether this time effect is caused by day-night differences, post-hoc analyses were performed comparing osteocalcin levels during day-time (10:00 – 22:00) with night-time (02:00 – 06:00). Men had a mean (95% CI) difference between day and night of 2.3 (1.4 – 3.3) µg/L, with significantly ($P < 0.001$) lower mean (SD) osteocalcin levels of 17.1 (5.1) µg/L during the day compared to the night (19.5 (5.8) µg/L). Also women had lower osteocalcin levels during the day (20.5 (6.7) µg/L) compared to the night (22.0 (7.0) µg/L) with a mean (95% CI) difference of 1.4 (0.5 – 2.4) µg/L ($P = 0.01$). However, no significant difference between men and women was observed ($F(1,35) = 2.5$, $P = 0.13$), nor a Time by Sex interaction ($F(3.9,135.9) = 1.2$, $P = 0.29$). The overall fit of the cosinor model was not significant ($P = 0.13$).

P1NP

24-h mean P1NP levels were slightly higher in women than in men, although not significantly (Table 2). By visual inspection, we observed that levels of P1NP (Figure 1c) were relatively constant over 24 h, but exhibited a small linear increase during 24 h with the highest point in the night at 02:00. Mean P1NP levels differed significantly between time points (F(4.1,139.0) = 7.3, $P < 0.001$), with a trend to higher levels in women compared to men (F(1,34) = 3.5, $P = 0.07$). However, no Time by Sex interaction was observed (F(4.1,139.0) = 0.5, $P = 0.75$). Men had significantly ($P = 0.001$) higher mean (SD) P1NP levels in the night (42.5 (14.8) ng/mL) than during the day (39.8 (13.7) ng/mL) with a mean difference (95% CI) of 2.7 (1.3 – 4.0) ng/mL. Levels of P1NP did not differ significantly ($P = 0.12$) between day (50.4 (15.5) ng/mL) and night (51.8 (15.6) ng/mL) in women. Post-hoc *t*-tests indicated that women had significantly higher levels of P1NP levels than men at 10:00 ($t_{df} = 2.1_{35}$, $P = 0.04$), 14:00 ($t_{df} = 2.1_{35}$, $P = 0.045$), and 22:00 ($t_{df} = 2.3_{35}$, $P = 0.03$). There was no significant fit of the cosinor model ($P = 0.59$).

Sclerostin

Table 2 presents no significant difference between men and women in their 24-h mean sclerostin levels. Over 24 h, sclerostin levels (Figure 1d) increased in men with its highest point at 18:00 followed by a decrease. In women, levels fluctuated over the day with peaks at 10:00, 18:00 and 02:00. Levels of sclerostin were not significantly different between men and women at any of the time points sampled. Furthermore, sclerostin levels differed significantly between time points (F(4.0,139.1) = 2.7, $P = 0.04$) with no difference between men and women (F(1,35) = 0.25, $P = 0.62$) and no Time by Sex interaction (F(4.0,139.1) = 0.6, $P = 0.67$). The cosinor model did not significantly fit the sclerostin levels ($P = 0.30$).

DKK1

24-h mean DKK1 levels did not differ between men and women (Table 2). DKK1 (Figure 1e) was relatively constant during daytime with a significant increase in the night for men. In women, mean DKK1 levels were also relatively stable over 24 h, except at 18:00, when there was a sudden increase. However, when individual 24-h profiles of DKK1 were plotted (see Supplementary Figure S1) and visually inspected, DKK1 exhibited a large variation between and within subjects. Neither the within-subjects factor Time was significant (F(3.3,108.0) = 2.2, $P = 0.09$), nor the between-subjects factor Sex (F(1,33) = 2.4, $P = 0.13$). However, there was a significant interaction between time and sex (F(3.3,108.0) = 3.0, $P = 0.03$). Post-hoc *t*-tests indicated that women had higher DKK1 levels at 10:00

(t_{df} =2.8$_{33}$, P = 0.01) and at 18:00 (t_{df} =2.6$_{33}$, P = 0.01) compared to men. The overall fit of the cosinor model was not significant (P = 0.51).

For all bone markers, results did not materially change after adding offspring-partner status as a between-subjects factor in the analyses.

DISCUSSION

This study is the first which simultaneously measured the 24-h trajectories of the five bone markers CTX, osteocalcin, P1NP, sclerostin, and DKK1 in the same participants, comprising healthy older men and women. Moreover, DKK1 levels had not been measured over time before and sclerostin levels had not yet been measured over time in healthy older women. In line with previous findings, we observed that CTX had a circadian rhythm with its nadir in the afternoon and its peak in the late night in both men and women. Although the timing of the circadian rhythm was similar between men and women, we are the first to observe a statistically significant larger CTX amplitude in women than in men. Furthermore, in line with previous findings, we observed that the 24-h profile of osteocalcin exhibited higher levels during night-time compared to daytime in both men and women. However, no significant circadian rhythm for osteocalcin was identified when a strict cosinor function was fitted. For P1NP levels we observed a small but significant increase in the night in men, but not in women. Sclerostin did not demonstrate a clear circadian rhythm, but levels differed between time points in both men and women. We did not observe a clear rhythm in DKK1 over 24 h in neither men nor women.

The circadian rhythm of CTX with its peak around 05:21 and 05:59 for men and women respectively was similar to that observed in other studies [3-6, 18]. We observed a difference between older men and women in the amplitude of CTX over 24 h, with a larger amplitude in women than in men. This was in contrast to other studies, which have shown that the circadian rhythm was independent of sex [11, 18]. The circadian rhythm of serum CTX is strongly influenced by fasting and food intake, with an increase in CTX during fasting resulting in a lower variation over 24 h [18, 43, 44]. CTX levels decreased after intake of food, glucose, fat, and protein. This response is independent of sex or age [43, 45]. In response to food intake, glucagon-like peptide-2 (GLP-2) is secreted, which is a gastrointestinal hormone involved in the maintenance of intestinal epithelial morphology and function [46]. Injection of GLP-2 caused a dose-dependent reduction in CTX levels [45]. In general, CTX levels are higher in postmenopausal women than in men [15, 16]. Although not formally asked, based on the age range of women and the higher levels in

women than in men, we assume (most) women in our study are postmenopausal. Both men and women received six bottles of Nutridrink consisting of 1800 kcal during the day. In general, women need fewer calories than men, so it could be that food intake caused a stronger reduction during the day in women than in men. Although unlikely, this could explain the nonexistence of a difference in levels between men and women during day time, and also the bigger amplitude in women than in men. However, we cannot exclude the possibility of a chance finding. Studies have shown that ethnicity, low bone mass, bed rest of 5 days, absence of a circadian rhythm of cortisol, absence of a light-dark cycle (blindness), or circadian disruption and sleep restriction did not influence the circadian rhythm of CTX [11, 18, 47]. Indeed, since we found similar circadian rhythm of CTX compared to literature, we assume that bed rest and potential sleep disturbances, possibly caused by performing blood sampling in the night, did not influence the circadian rhythm of CTX.

For osteocalcin, we found significantly higher levels during night-time compared to daytime in both men and women. When performing cosinor analysis, no significant circadian rhythm was observed. However, although cosinor analysis is often used to determine circadian rhythms, this model is very strict. If a curve does not fit into a cosine function, one would conclude that no circadian rhythm is observed. However, not all circadian rhythms fit into a cosine function. For example, a curve comprising every 24 h a sharp peak at the end of the night still displays a circadian rhythm but this does not fit into a cosine function. Therefore, we can conclude from our data that osteocalcin, although not detected with cosinor analysis, displays a circadian rhythm with its nadir during daytime and its peak in the night, which is in line with previous studies [5, 8, 11, 21-24]. No differences in 24-h profile of osteocalcin were found between men and women, which is in line with literature [11, 23]. Besides being released into the circulation during bone formation, osteocalcin fragments are also released into the circulation during bone resorption since it is part of the bone matrix. This might explain why osteocalcin has a similar circadian rhythm as CTX and has a stronger circadian rhythm than other bone formation markers [9, 48]. Studies have shown that the circadian rhythm of osteocalcin is highly dependent on the circadian variation in serum cortisol [49-52]. The circadian rhythm of cortisol is mostly influenced by the circadian clock, but cortisol levels also depend on other factors such as stress and activity [53]. First, participating in a clinical study is stressful; especially blood withdrawal during 24 h with limited movement could lead to stress and could influence the levels of cortisol. Furthermore, participants of our study performed several tests, including the Trier Social Stress Test and tests for cognitive function, on the day before the blood sampling and were wearing multiple sensors to

register physiological parameters during the study. Potentially, sleep disturbances caused by performing the continuous blood sampling could have influenced the cortisol rhythm causing less circadian variation of osteocalcin. Furthermore, participants had unusual food intake during the continuous blood sampling. Although participants received mixed meals containing all essential nutrients, their food intake consisted of liquid meals only. Literature showed that fasting did not significantly change the circadian rhythm of serum osteocalcin [43, 44, 54]. For osteocalcin levels, one study showed slightly lower osteocalcin levels in the fed state, while another study showed no effect of food intake or GLP-2 injection [45, 55]. Therefore, it is unlikely that the unusual food intake in the form of liquid meals has influenced the circadian rhythm of osteocalcin. The circadian rhythm of osteocalcin was not influenced by ethnicity, low bone mass, or by bed rest of 5 days [11, 21, 22, 24]. Therefore, since we found similar circadian rhythm of osteocalcin compared to literature, we assume also that bed rest and limitation in their movement and lack of activity did not influence the circadian rhythm of osteocalcin.

Literature on the circadian rhythm of P1NP is limiting and conflicting [6, 10, 11]. In our study, P1NP did not exhibit a clear circadian rhythm in neither men nor women, but P1NP levels were higher in the night than during the day in men. Also other studies found that day-night differences were less pronounced for markers of bone formation compared to bone resorption, reviewed by others [9, 56]. In general, P1NP levels were higher in women than in men, similar to findings from other studies [15, 16]. The participants were mostly bedbound and were limited in their movements during the 24 h in which the blood was withdrawn. Although studies have shown no effect of five days of bed rest on the circadian rhythm of CTX and osteocalcin, it could have influenced the 24-h profile of P1NP [18, 22]. However, the circadian rhythm of carboxyl-terminal propeptide of type 1 procollagen (P1CP), which is closely related to P1NP, was not influenced by five days of bed rest [22]. Furthermore, sleep disruption did not significantly change the circadian rhythm of P1NP [47]. Although the effect of fasting or food intake on the circadian rhythm of P1NP was not investigated, P1NP levels were somewhat lower in the fed state, and P1NP levels decreased mildly during an intravenous glucose tolerance test, but oral glucose ingestion did not have a significant effect on P1NP levels [55, 57, 58].

Sclerostin has only been measured once over time by other researchers, and no circadian rhythm of sclerostin was observed in men [6]. We confirmed this is men, but also in women we did not identify a circadian rhythm. However, sclerostin levels differed between time points in both men and women, so there seems to be an effect of time. Sleep disruption did not significantly change the circadian rhythm of sclerostin [47].

However, it is not known whether food intake, limited activity or 24-h bed rest may have influenced the circadian rhythm of sclerostin.

DKK1 levels have not been measured over time before. Our measurements of DKK1 displayed a large variation between participants, but we also see a large variation of DKK1 within participants. This variation within a participant over time cannot be explained by circadian variation since no circadian rhythm was detected nor a 24-h profile over time. It is not clear what the cause of this wide variation is. One explanation could be that DKK1 is secreted in a pulsatile fashion, similar to some other regulatory factors such as hormones. For example, when growth hormone (GH) was measured in the same participants in blood sampled every 10 min during 24 h, we could indeed assess its pulsatility. When GH would have been measured every 4 h, however, the individual variation would also be large [59]. DKK1 levels should be assessed more frequently than every 4 h to confirm this hypothesis. Another explanation could be that DKK1 is not bone specific, but also secreted by human preadipocytes and promotes adipogenesis [60]. A study showed that DKK1 is also expressed in platelets [36]. The activity of blood platelets varies over 24 h which could explain the variation in DKK1 levels [61]. However, the variation in platelet activity described in this study is much lower than the variation in DKK1 levels we have measured over 24 h. Moreover, blood platelets are intact in EDTA plasma, in contrast to serum, therefore DKK1 levels present in platelets did not contribute to the DKK1 levels present in plasma. It is unclear whether freeze-thaw cycles of the material could have influenced the results. Based on our previous hands-on experience with the assay, we do not have assumptions that the ELISA assay did not work correctly. This assay has been performed several times in our laboratory and results have been published before [38]. For DKK1, at a frequency of 4 h, we cannot reliably derive information on the absence or presence of the 24-h rhythm of DKK1.

Sclerostin and DKK1 are different types of bone markers than CTX, osteocalcin, and P1NP. While the latter are by-products of collagen breakdown or incorporated in the bone matrix, sclerostin and DKK1 have regulatory roles. Moreover, since osteocytes are highly sensitive to changes in calcium and phosphate levels and to mechanical loading, levels of their secreted regulatory proteins will be more susceptible to internal and external factors [12]. Indeed, sclerostin and DKK1 levels changed after mechanical (un)loading [62]. This might explain why sclerostin and DKK1 levels demonstrate large variations over 24 h and did not display similar 24-h profiles as other bone markers.

Limitations of our study are the unusual (not similar to daily-living) study conditions, including staying in bed with limited movement during the 24 h in the research centre, food intake which only consisted of liquid meals, and sleep which was potentially disturbed by the continuous blood sampling. This could have influenced the results. Furthermore, bone mineral density was not assessed, since bone health was not a primary objective of this study. Although the study conditions are not similar to daily-living conditions, the conditions were standardized for all participants, so individuals were studied in a comparable research setting which is a strength of this study. Nevertheless, standardisation during the study period only does not completely rule out the influence of possible individual factors on the 24-h profiles of bone markers. This study is the first to measure all these bone markers during 24 h in 37 participants, a relatively large study population for investigating the circadian rhythm of bone markers. Future studies could measure more samples per participants to be able to determine the exact timing of the circadian rhythm of the bone markers. Also, a period longer than 24 h could determine the stability of the rhythm over several days.

In conclusion, for DKK1 as measured with a frequency of 4 h in this study, no reliable conclusion on the absence or presence of a 24-h rhythm can be drawn because of the large intraindividual variation. Furthermore, when measuring CTX, osteocalcin, P1NP, or sclerostin either in clinical practice or in a research setting, one should consider the 24-h profiles of these bone markers. It has to be taken into account that not only CTX and osteocalcin have a circadian rhythm but P1NP and sclerostin levels, although not exhibiting a circadian rhythm, vary as well during the day. Although levels differed between men and women, timing of the 24-h rhythm of all bone markers did not differ between men and women. Potentially, sex hormones predominantly influence levels of bone markers, but not interfere with the timing of the 24-h rhythm. Further research could focus on the association between sex hormones and the circadian rhythm of bone markers.

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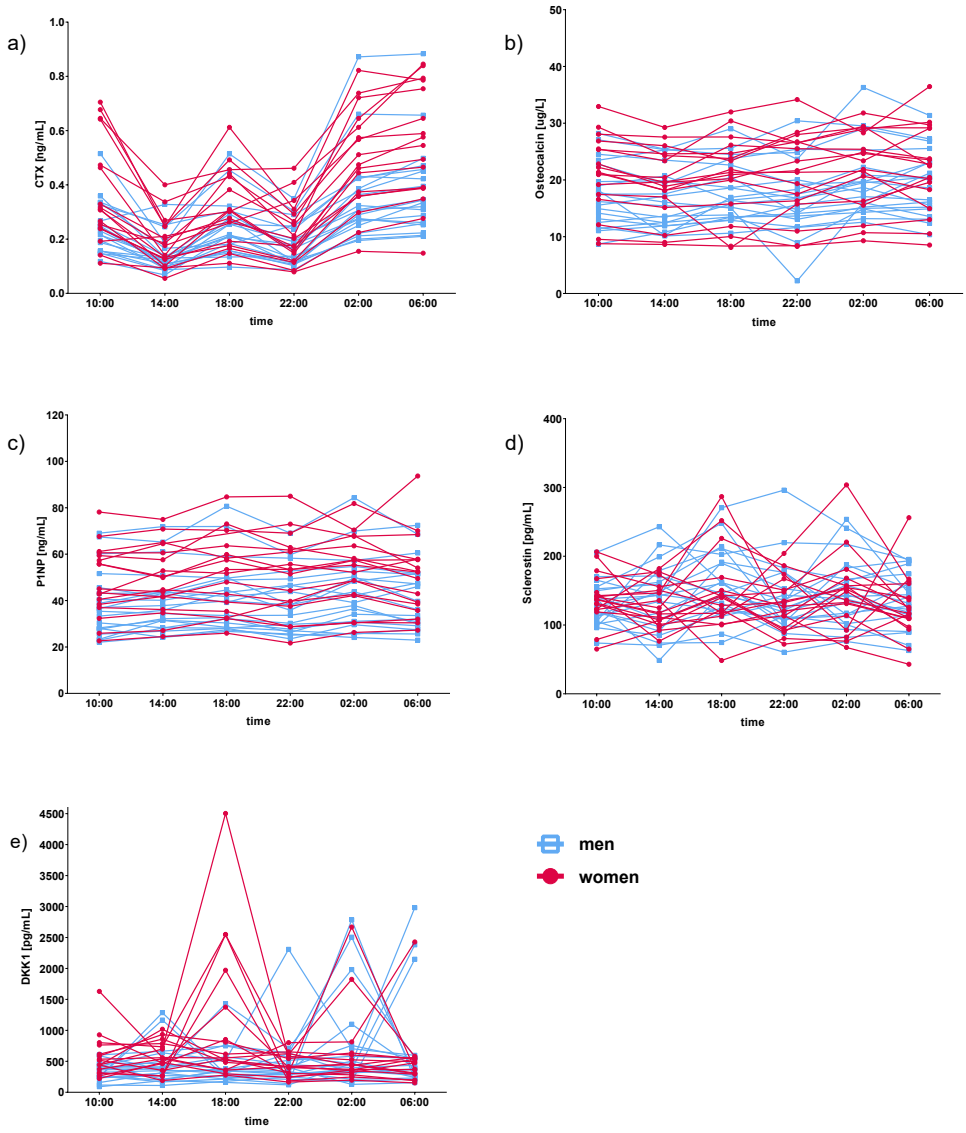
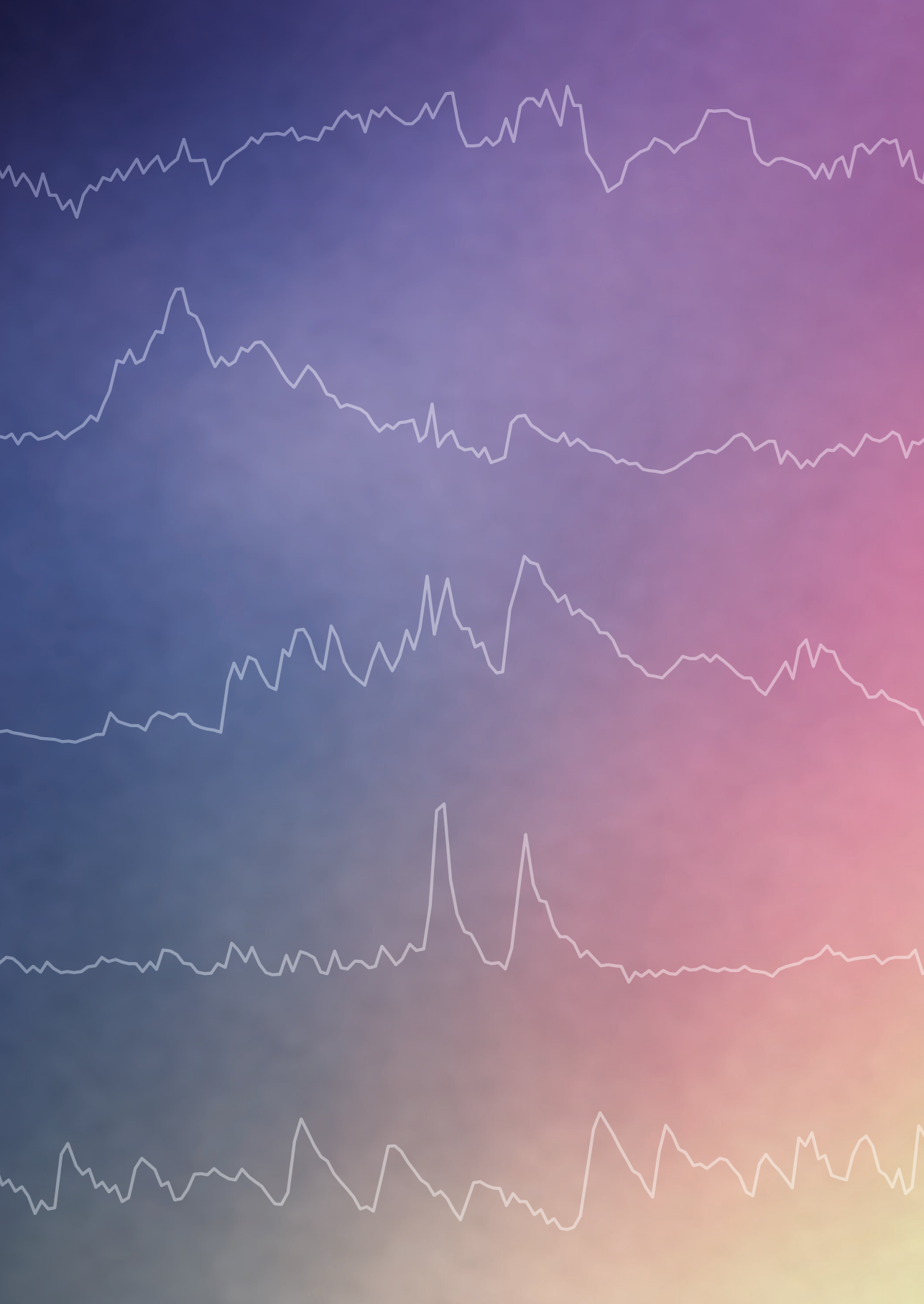
SUPPLEMENTARY DATA



Supplementary Figure S1. Individual 24-hour profiles of bone markers.

Individual 24-hour profiles of a) C-terminal cross-linked telopeptide of type 1 collagen (CTX), b) osteocalcin, c) N-terminal propeptide of type 1 procollagen (P1NP), d) sclerostin, and e) Dickkopf-related protein 1 (DKK1) are presented every 4 hours starting at 10:00 for men (blue, light) and women (pink, dark).



7

General discussion

GENERAL DISCUSSION

Ageing

Ageing has been described as "a progressive, generalized impairment of function, resulting in an increasing vulnerability to environmental challenge and a growing risk of disease and death" [1]. It is hypothesized that due to evolved limitations in maintenance and repair, molecular damage will accumulate gradually, and interfere with the integrity of cells and tissues, thus driving functional decline and risk of disease and death. From this hypothesis, it follows that the rate of ageing is largely determined by the balance between damage accumulation and defence and repair mechanisms. Many external and internal factors can affect this balance. Among these, neuro-endocrine pathways, which are centrally regulated by the brain, are thought to play an important regulatory role [2]. The common goal of these neuro-endocrine pathways is to maintain homeostasis in the body by detecting changes in the environment and by orchestrating a coordinated physiological response to these signals. In this context, one of the best known neuro-endocrine pathways is the insulin/insulin-like growth factor 1 (IGF-1) signalling (IIS) pathway. This pathway regulates metabolism and growth in invertebrates, including yeast, *Caenorhabditis Elegans* (*C. Elegans*), and *Drosophila melanogaster*, but also in mammals, including mice and human. In invertebrates, central regulation of the IIS pathway occurs via the production of different insulin/IGF-1 like neuropeptides. In mammals, the IIS pathway is divided into two separate, but partly overlapping, signalling pathways; the insulin signalling pathway regulating primarily glucose and lipid metabolism and the growth hormone (GH)/IGF-1 pathway, regulating primarily linear and cellular growth. The upstream regulatory hypothalamic factors and the secretion of GH from the pituitary are present in mammals, but not in invertebrates. Despite differences in complexity and regulation, the core downstream components of the IIS pathway are conserved between invertebrates and mammals.

Neuro-endocrine control of ageing in animal models

Reduced insulin/IGF-1 signalling has been consistently associated with longevity in various model organisms, from yeast to mice [3-10]. For example, the lifespan of *C. Elegans* can be prolonged from 2-3 weeks up to 6 months when mutations in the daf-2 gene, which is homologous to the insulin receptor, were made [11]. Actually, mutations in almost all core components of the IIS are associated with extended lifespan in *C. Elegans*. Also, a loss of function mutation in the insulin receptor of *Drosophila* has been associated with prolonged lifespan [10]. As another example, the health- and lifespan of mice lacking functional growth hormone releasing hormone (GHRH) or GH receptors is prolonged

compared with wildtype controls [12, 13]. In these animal models, the prolonged lifespan comes at the costs of reduced reproductive capacity and growth, indicating the existence of trade-offs. These studies indicate that not only the IIS pathway is evolutionary conserved, but that also the link between reduced insulin/IGF-1 signalling and longevity is an evolutionary conserved mechanism. The ligands, receptors and downstream signalling molecules that were found to have a major impact on longevity in yeast, fruit flies, and worms are homologous to proteins from the insulin/IGF-1 pathway in mammals. Moreover, these conserved proteins are regulated by GH in mammals.

Neuro-endocrine control of human ageing

The studies in animal models demonstrated the important role of neuro-endocrine pathways in the regulation of ageing. However, the relations between neuro-endocrine parameters and the human ageing process are complex and not fully understood. The main neuro-endocrine pathways in human are the hypothalamic-pituitary-target gland axes, consisting of the somatotropic, thyroid, adrenal, gonadal, and prolactin axes (Figure 1). In previous studies with participants of the Switchbox Study, a sub-study of the Leiden Longevity Study, associations between familial longevity and hormones of the hypothalamic-pituitary-thyroid (HPT) and the hypothalamic-pituitary-adrenal (HPA) axes have been investigated. It was found that offspring of long-lived families and their partners had no major differences in HPA axis activity, but the offspring had higher total thyroid-stimulating hormone (TSH) secretion compared to partners [14, 15]. Whether familial longevity is also associated with altered endocrine features in other hypothalamic-pituitary-target gland axes or their interplay had not been investigated yet. Since offspring and partners had similar thyroid hormone levels, resting metabolic rate, and core body temperature, we hypothesized that pleiotropic effects of the HPT axis cause the favourable effects on lifespan, for example on tissue maintenance. Interestingly, preliminary data indicated that levels of biomarkers of bone turnover differed between offspring of long-lived families and partners. In this thesis, we focussed on the roles of the hypothalamic-pituitary-somatotropic and hypothalamic-pituitary-gonadal axes in familial longevity, the interrelationships between hormones of the hypothalamic-pituitary-target gland axes, and the 24-h profiles of bone turnover markers in healthy older subjects.

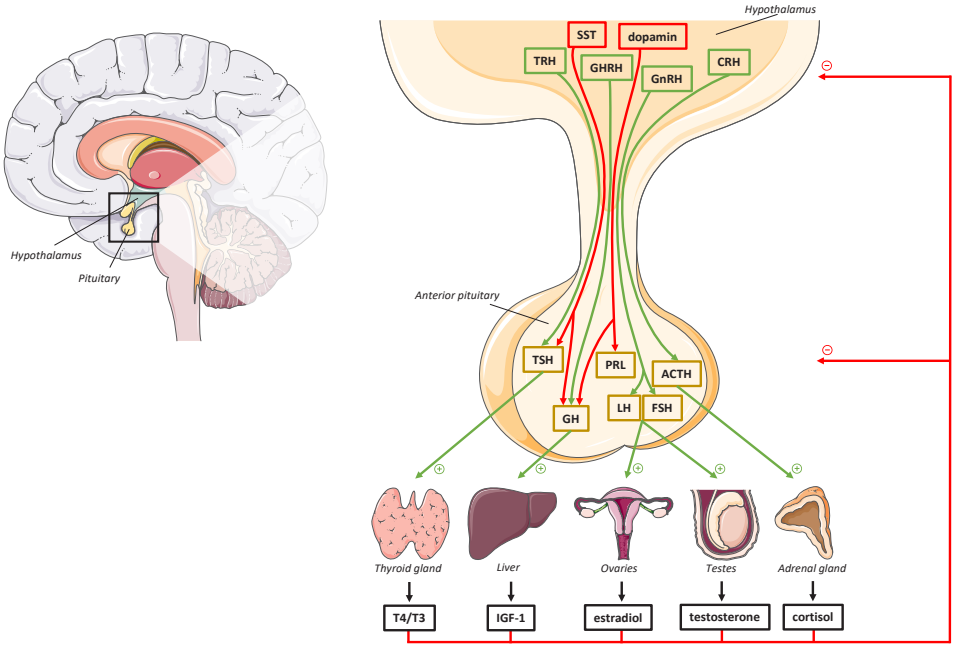


Figure 1. Hypothalamic-pituitary-target gland axes.

Schematic representation of the (regulation of the) human hypothalamic-pituitary-target gland axes; hypothalamic-pituitary-thyroid axis, hypothalamic-pituitary-somatotropic axis, hypothalamic-pituitary-gonodal axis, hypothalamic-pituitary-prolactin axis, and hypothalamic-pituitary-adrenal axis. The green arrows indicate the stimulation of hormone secretion and the red arrows the inhibition of hormone secretion. Figures of tissues/organs were adapted from Servier Medical Art, *https://smart.servier.com/*. Abbreviations:

ACTH: adrenocorticotropic hormone
CRH: corticotropin-releasing hormone
FSH: follicle-stimulating hormone
GH: growth hormone
GHRH: GH-releasing hormone
GnRH: gonadotropin-releasing hormone
IGF-1: insulin-like growth factor 1
LH: luteinizing hormone
PRL: prolactin
SST: somatostatin
T3: triiodothyronine
T4: thyroxine
TRH: thyrotropin-releasing hormone
TSH: thyroid-stimulating hormone

Hypothalamic-pituitary-somatotropic axis and familial longevity

The hypothalamic-pituitary-somatotropic axis (GH/IGF-1 axis) consists of hypothalamic growth hormone releasing hormone (GHRH), GH from the pituitary and IGF-1 mainly produced by the liver (Figure 1). In **Chapter 2** of this thesis, we investigated whether circulating IGF-1 axis parameters were associated with old age survival and functional status in nonagenarian siblings from the LLS. Studies on circulating IGF-1 and mortality have yielded inconclusive results since both low and high IGF-1 concentrations are associated with increased mortality. It is important to note that circulatory levels of IGF-1 predominantly reflect IGF-1 produced by the liver. Also other tissues produce IGF-1, which

is mainly GH-independent, and these paracrine IGF-1 concentrations are likely to be much higher than plasma concentrations. Unfortunately, these are difficult, if not impossible, to measure in human. Furthermore, GH was not included in this study since GH is secreted in a pulsatile manner and has a short half-life and should therefore be measured in blood which was sampled frequently over the day to obtain reliable information. This type of study was however not possible in this study population. Circulatory IGF-1 is more stable over a 24-h period than GH. Both GH and IGF-1 are bound to binding proteins, but only 40% of GH is bound to binding proteins while 99% of IGF-1 is bound to binding proteins, of which IGFBP3 is most abundant, which largely explains the longer half-life of IGF-1. In Chapter 2, it was observed that lower IGF-1/IGFBP3 molar ratios, which are indicative of reduced IGF-1 availability, were associated with improved survival. Furthermore, nonagenarians in the lowest quartile of IGF-1/IGFBP3 ratios had the highest scores for both Activities of Daily Living (ADL) scales and Instrumental ADL scales, indicating better functional status. It is unknown whether these long-lived nonagenarians only had reduced IGF-1 availability at old age or whether these nonagenarians already had reduced IGF-1 availability at younger age. Unfortunately, we do not have circulating levels of their IGF-1 parameters at young age, but we did include their offspring in the Switchbox Study, of which the results are discussed in **Chapter 3**.

In Chapter 3, we found an association between lower GH secretion and familial longevity. No significant differences were observed in circulating levels of IGF-1 and IGFBP3 between offspring and controls, although these tended to be somewhat lower in the offspring. As the study discussed in Chapter 2, this study was also a cross-sectional study, so we do not know whether these healthy older individuals had a reduction in their GH secretion at older age or already had low GH secretion at younger age. These results could indicate that GH secretion is more important for longevity than circulating IGF-1. In support of this hypothesis, the impact of disrupting GH signalling on longevity is larger than the impact of disrupting IGF-1 signalling or events downstream from IGF-1 receptors in laboratory mice [16].

Although GH and IGF-1 are part of the same signalling pathway and stimulate growth, the short-term effects of GH and IGF-1 are complementary. Acute effects of GH can be seen as anti-insulinogenic effects, since GH inhibits glucose uptake by muscle, stimulates lipolysis in adipose tissue, and stimulates gluconeogenesis in the liver, while IGF-1 has similar effects as insulin, especially on the muscle by stimulating protein synthesis and the uptake of glucose and amino acids. Moreover, GH has no direct effect on longitudinal growth, but only indirect via IGF-1. This could explain the fact that the offspring had

similar height as their partners, since offspring had lower GH secretion, but similar IGF-1 levels compared to partners. The lifespan-extending mutations in yeast, fruit flies, worms and mice lead to an accumulation of glycogen and/or fat. Surprisingly, although all these mice are obese, mice with reduced GH action are insulin sensitive and the mice with reduced IGF-1 action are insulin resistant. This observation supports the fact that GH and IGF-1 also have opposite/independent effects besides their similar effects.

The mechanisms by which reduced GH/IGF-1 signalling is leading to ageing and longevity have mainly been investigated in mouse models and reviewed by Bartke *et al.* [8, 17, 18]. It was among others observed that mice with reduced GH signals had beneficial alterations in their anti-oxidant defence mechanisms, stress resistance, and oxidative damage. Furthermore, reduced GH signals led to increased fatty acid oxidation and insulin sensitivity, but a decrease in both IGF-1 and mTOR signalling. Moreover, the number of senescent cells and inflammatory processes were decreased in mice with reduced GH signalling. All these processes are integrated in an interactive network that is associated with decreased risk of cancer, diabetes, and other age-related diseases, and also with longevity. Whether the same mechanisms will be present in humans is not completely clear. Unravelling these mechanisms in human could lead to potential starting points for intervening in the ageing process and hopefully eventually lead to healthy human ageing.

Studies in patients with a genetic alteration in the GH/IGF-1 pathway causing GH-deficiency or GH-resistance show contradictory results on health and longevity. Laron syndrome dwarfs with GH receptor gene mutations causing congenital growth hormone resistance were found to be protected against cancer and have relatively long lifespans [19, 20]. However, the lifespan of a group of 11 patients with untreated late onset isolated GH deficiency in Switzerland were relatively short compared to their unaffected siblings and these patients had an increased risk of cardiovascular disease [21]. Patients and animal models carry mutations that have a large impact on GH action, while subjects from the Switchbox Study do not have this extreme phenotype but rather display a more subtle alteration in their GH/IGF-1 axis. It is therefore an interesting observation that already a small difference in GH secretion is associated with familial longevity. The association between the GH/IGF-1 axis and familial longevity have also been investigated in other human studies. For example, a study in Italy showed that the IGF-1 bioactivity, levels of total IGF-1, and the IGF-1/IGFBP3 ratio were lower in the offspring of centenarians compared to an age-matched control group [22]. Furthermore, the female offspring of Askenazi Jewish centenarians displayed several signs of relative IGF-1 resistance, including higher serum IGF-1 levels and a smaller stature [23]. Since these

studies were cross-sectional and group-based, it makes it more difficult to unravel mechanisms underlying the link between reduced GH action and longevity in this population. It is important to keep this in mind when comparing (our) observations in humans to the studies in animal models.

Taken together, these observations led to the strong and convincing conclusion that reduced GH/IGF-1 signalling leads to a prolonged health- and lifespan. However, since GH levels decline with age and because of beneficial effects of GH on muscle and adipose tissue, reduced GH levels can also be viewed as a result, or even a cause, of the ageing phenotype. In general, it is assumed that most of the age-related changes are not beneficial for health- and lifespan. For example, ageing is also associated with a decline in cognitive functioning, mobility, and insulin sensitivity. However, anything that is beneficial at young age does not necessarily have to be beneficial at old age as well, and maybe it is sometimes even the other way around. Some changes with age could actually be beneficial, especially if these reflect an adaptive response of the body to survive, since these survival responses could lead to prolonged health- and lifespan. The decline in circulating GH and IGF-1 levels could reflect such a survival response and it could indicate that more energy is invested in maintenance and repair instead of in growth. However, studies in mice showed that especially reduced GH signalling during early life is important for longevity. For example, exposure to GH treatment early in life reduced longevity of dwarf mice [24].

Besides lower GH secretion, we also found in Chapter 3 that GH secretion was more tightly controlled in the offspring of long-lived families compared with their partners. This could indicate that the timing and regulation of a hormone is just as, or even more, important than total hormone levels. When the right amount of a hormone is secreted at the right moment, no waste of hormone resources occurs. More importantly, saving resources of the body could potentially lead to a longer health- and lifespan since it can be argued that less energy is invested in growth and development and more energy can be invested in maintenance and repair. This is in line with our hypothesis that longevity is associated with a prolonged ability to preserve the optimal balance between investments in growth and development versus maintenance and repair throughout life. Both low GH secretion as well as its tighter regulation could lead to preserved hormone resources. To test this hypothesis in future studies, the association between GH/IGF-1 signalling and biomarkers of tissue maintenance could be investigated.

Hypothalamic-pituitary-gonadal axis and familial longevity

Another hormonal axis is the hypothalamic-pituitary-gonadal (HPG) axis, which consists of hypothalamic gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary, and gonadal secretion of testosterone in men and oestrogen in women (Figure 1). As GH and IGF-1 levels, testosterone and oestrogen levels also decrease with age, with an acceleration after menopause in women. A later age of menopause was associated with reduced female mortality [25]. In model organisms, many long-lived mutants have a reduced reproductive output. Also in humans, decreased reproduction was found to associate with exceptional human longevity in both men and women [26]. Longevity inducing interventions, such as fasting, led to a decline in LH secretory burst mass and testosterone concentrations and to an increase in LH release pattern orderliness [27, 28]. In **Chapter 4**, we investigated in healthy male middle-aged participants whether familial longevity is associated with altered LH and testosterone secretion. We found no major differences in features of the HPG axis between male offspring of long-lived families and the control group. This could be related to the selection criteria of the study population, which were based on the presence of at least two long-lived siblings, so we may have selected on fertility, and the strict inclusion criteria on health. We also did not observe differences in single measurements of oestradiol and prolactin in a 24-h pool between offspring of long-lived families and controls, neither in men nor in women. Although this was a relatively small study, and therefore possibly underpowered, these results suggest that the HPG axis is not involved in familial longevity.

Interrelationships between hormones of the hypothalamic-pituitary-target gland axes

Up to now, most research on the associations between neuro-endocrine pathways and longevity is performed for each neuro-endocrine pathway separately. The interplay between neuro-endocrine pathways have rarely been addressed, which is surprising since neuro-endocrine pathways are highly linked with each other. For example, anterior pituitary cells share the same embryonic origin; the anterior pituitary is derived from oral ectoderm [29]. During the embryonic development of the anterior pituitary, specific genes direct the cells toward a particular fate. For lactotrophs, somatotrophs, and thyrotrophs, the same genes are involved in their development until the final differentiation. This means that lactotrophs, somatotrophs, and thyrotrophs largely share the same developmental cascade. Moreover, there is evidence for crosstalk between pituitary cells [30]; studies in rats showed that there is functional overlap between the different anterior pituitary cell types and many anterior pituitary cells respond to more than one

hypothalamic-releasing hormone [31, 32]. Furthermore, the main goal of these neuro-endocrine pathways is to maintain homeostasis in the body by detecting changes in the environment and by responding to these signals. It is therefore very likely that these pathways interact and/or have shared actions. When investigating each hormonal axis separately, we found in the Switchbox Study that altered endocrine features of the thyroid and somatotropic axes were associated with familial longevity. These associations might reflect separate mechanisms, but these hormonal changes could also be synchronized with each other and their concerted impact might be larger than the sum of their individual impacts on ageing and longevity. Also in other systems and organs of the body, interplay, interaction, and networks are highly important for maintenance of homeostasis and proper functioning. Additionally, the current understanding is that all hormones in a certain hormonal axis have the same primary actions. However, for example within the somatotropic axis this is not the case since GH and IGF-1 have diverse effects. This could therefore also be the case for the other hypothalamic-pituitary-target gland axes. The interrelationship between TSH and adrenocorticotropic hormone (ACTH) could for example be different from the interrelationship between TSH and cortisol.

Therefore, in **Chapter 5** of this thesis, we investigated the interrelationships between hormones of hypothalamic-pituitary-target gland axes within axes as well as between axes. We confirmed that within interlinked hormonal axes, ACTH and cortisol concentrations are correlated. The correlations found between hormonal axes, so between cortisol and TSH concentrations, between TSH and GH concentrations, and the joint pattern synchrony between GH and cortisol are indications that there is interplay between hormonal axes in healthy older individuals. These interconnected hormones are also the hormones mostly affected by survival responses, including calorie restriction. However, no major differences in the interrelationships between hormones were found between offspring of long-lived families and partners. This could indicate that this interplay between hormones is crucial for survival and if this interconnection would disappear, it would lead to illness. Participants in this study were selected based on their health status which resulted in a group of healthy older individuals and this could have influenced the results. The strong correlation found between GH and TSH concentrations measured simultaneously, which were the only two hormones of which the secretion differed between offspring and partners, could indicate that a common upstream regulator may regulate both hormones. The increased TSH secretion and reduced GH secretion in offspring of long-lived families could be pleiotropic effects of this upstream regulator while this regulator influences longevity via another mechanism.

Biomarkers of tissue maintenance

The mechanisms by which hormones of the hypothalamic-pituitary-target gland axes are associated with longevity still need to be elucidated. We hypothesize that (the interplay of) hormones of the different hypothalamic-pituitary-target gland axes are key regulators in adjusting the balance between investments in growth, development, and reproduction versus maintenance and repair to its optimal state (see Figure 2). The optimal balance between these processes will be different for the different phases of the life cycle and we hypothesize that longevity is associated with a prolonged ability to preserve an optimal balance throughout the different phases of life. To test this hypothesis, valid biomarkers of tissue maintenance should be assessed, and it needs to be determined whether biomarkers of tissue maintenance are indeed early markers of the ageing process. Subsequently, the association between hormones and biomarkers of tissue maintenance should be investigated.

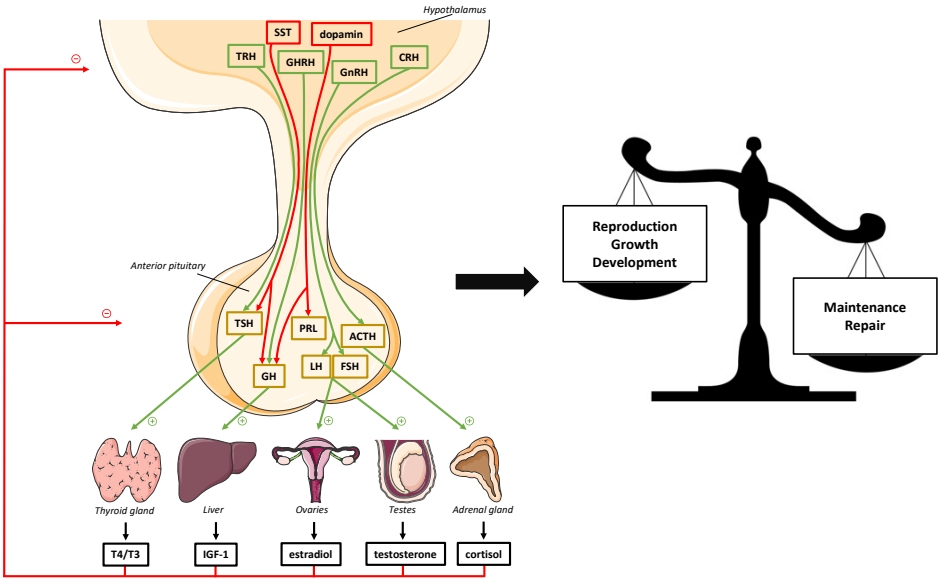


Figure 2. Hypothesis: hormones of the hypothalamic-pituitary-target gland axes regulate the balance between investments in growth, development, and reproduction versus maintenance and repair.

A review on a proposed panel of biomarkers of bone, cartilage, muscle, and brain tissue maintenance has been written during this PhD project (manuscript in press, not included in this thesis). At this moment it is unknown whether it is beneficial to have elevated levels of biomarkers of tissue maintenance or reduced levels (assuming that the amount of damage is equal). Higher levels could indicate that the tissue is capable to regenerate but

it could also indicate that eventually the capacity of regeneration will decrease faster, leading to an earlier onset of age-related functional decline. Lower levels could indicate that many cells are already in senescence, so fewer cells are able to regenerate, which is undesirable. On the other hand, lower levels could also indicate that the tissue is only regenerating itself when this is necessary leading to prolonged ability for maintenance and repair, thus increased lifespan. Moreover, it could be that it is beneficial to have elevated levels at young age but reduced levels at old age. To determine this, the proposed biomarkers could for example be measured at different timepoints in their life in humans with different rates of ageing, including people showing delayed biological ageing compared to accelerated biological ageing, or offspring of long-living families compared to age-matched controls. However, before validated biomarkers can be generally used in research or the clinic, measurement of these biomarkers should also be standardized [33]. Whether reduced or elevated levels of biomarkers of tissue maintenance are beneficial might also be dependent on the context, so on the (micro)environment, communication with other tissues and cells, and/or circulating factors. Therefore, when measuring biomarkers of tissue maintenance, one should consider to include other regulatory factors to get a more comprehensive picture. Moreover, these regulating factors might even be more informative than a single biomarker of tissue maintenance. Therefore, besides validating proposed biomarkers of tissue maintenance, future research should include identification of circulating factors crucial for tissue maintenance.

One other important aspect that needs to be determined before the association between hormones and biomarkers of tissue maintenance could be investigated, is the circadian rhythm of these biomarkers. If these biomarkers vary over the day, these biomarkers should be measured frequently over the day to obtain reliable data. In **Chapter 6** of this thesis, we confirmed the circadian rhythm of the bone resorption marker C-terminal cross-linked telopeptide of type 1 collagen (CTX) and found that postmenopausal women had a larger amplitude than men. Osteocalcin, which is mostly a biomarker of bone formation, showed higher levels during night-time compared to day-time in both men and women. For the bone formation marker N-terminal propeptide of type 1 procollagen (P1NP) levels we observed a small but significant increase in the night in men. Sclerostin and Dickkopf-related protein 1 (DKK1), which are negative regulators of bone formation, did not show a circadian rhythm, but sclerostin levels differed between time points. Because of the large intraindividual variation, DKK1 as measured in this study cannot be considered a reliable marker for diagnostic or research purposes.

Hormone replacement therapy

It is thought that the age-related changes in hormone levels, including GH, TSH and sex hormones, are possible contributors to many problems in older persons. For example, it even has been suggested that since the ageing phenotype has similarities with the clinical picture of patients with GH-deficiency, that the age-related decline is the cause of the ageing phenotype. It is therefore an ongoing debate whether there should be replacement therapy for GH in elderly to prevent age-related loss in functioning. Although GH therapy did have small beneficial effects on body composition, overall it did not result in increased muscle strength or bone mineral density. Moreover, several randomized controlled trials have shown that GH therapy in healthy elderly is associated with increased rates of various side effects [34].

Also the prevalence of subclinical hypothyroidism, which is defined as an elevated TSH level together with an fT4 level within the normal range, is increasing with ageing [35]. It has been suggested that levothyroxine supplementation would provide clinical benefits in these older persons with subclinical hypothyroidism. However, a recent randomized clinical trial in older persons with subclinical hypothyroidism showed that although TSH levels in participants receiving levothyroxine treatment decreased, there was no beneficial effect on their thyroid-related symptoms compared to the placebo group [36]. Also a systematic review and meta-analysis provided evidence that the use of thyroid hormone therapy was not associated with improvements in general quality of life or thyroid-related symptoms [37]. The offspring of long-lived families from the Switchbox study also had, albeit still in the normal range, higher TSH secretion compared to their partners [14]. Also the offspring of Ashkenazi Jewish centenarians and these centenarians themselves had elevated TSH levels compared to controls [38, 39]. These studies support the idea that elevated TSH are not necessarily unfavourable.

The decline in oestrogen with ageing in women, most predominantly during menopause, is one of the risk factors for osteoporosis. Therefore, hormone replacement therapy (HRT) is often given to postmenopausal women to lower their risk of developing osteoporosis. Although HRT was indeed associated with decreased fracture risk, it was also associated with adverse effects including an increased risk of certain types of cancer [40, 41]. Also testosterone replacement therapy is a popular intervention; testosterone replacement therapy use has been three to four times increased between 2003 and 2013 in the United States [42]. Meta-analyses showed that lean mass and health-related quality of life increased in older men using testosterone therapy [43, 44] but the risk of prostate cancer and haematocrit were among others increased compared to the placebo group [45].

Circulatory levels do not always represent tissue-specific levels of hormones. For example, many tissues produce paracrine IGF-1 that does not contribute to circulating IGF-1. Interestingly, in Ames dwarf mice with reduced GH secretion, brain IGF-1 levels were elevated compared to control mice [46]. This could indicate that tissue-specific levels of hormones are more important for ageing and longevity than circulatory levels. Moreover, tissue-specific hormone supplementation could lead to health benefits in contrast to general hormone therapy.

Future perspectives

Future studies should aim to disentangle underlying mechanisms of the altered endocrine features of the thyroid and somatotropic axes in human longevity. One question is whether longevity is primarily achieved via the thyroid axis or somatotropic axis or whether both hormonal axes collaborate in achieving longevity. The somatotropic axis has been more consistently associated with longevity in animal models than the thyroid axis. However, studies are primarily performed in mice with a central TSH-deficiency leading to hypothyroidism, but the TSH secretion was higher, although still in the normal range, in offspring of long-lived families compared with the control group while their thyroid hormone levels were similar. To our knowledge, no studies have been performed assessing the lifespan in animal models with increased TSH secretion but with normal thyroid hormone levels. In this thesis we showed that TSH and GH concentrations were positively correlated without any delay. This could indicate that these hormones collaborate in achieving longevity. However, as mentioned in the discussion, another possibility is that both TSH and GH secretion are not causally related to longevity, but that the altered secretion of GH and TSH are caused by a common upstream regulator. The increased TSH secretion and reduced GH secretion in offspring of long-lived families could be pleiotropic effects of this upstream regulator while this regulator influences longevity via another mechanism. However, no major differences in the interrelationships between hormones were found between offspring of long-lived families and partners.

Since the Switchbox Study was an observational cross-sectional study, direct effects between hormones could not be determined. For this purpose, intervention studies in which a certain pituitary (or hypothalamic) hormone is administrated and other hormones are frequently measured need to be performed. In order to investigate the importance of the interplay of hormones in ageing and longevity, future studies could include offspring of long-lived families and partners with less strict exclusion criteria on health. Ideally, participants would be followed over time to determine changes in both hormone secretion and hormonal interrelationships with ageing.

Our main hypothesis is that the underlying mechanism of the relationship between pituitary hormones and longevity is tissue maintenance. To test this hypothesis, the effect of pituitary hormones on tissue maintenance should be assessed. For this, as discussed earlier in this discussion, biomarkers of maintenance of tissues that are crucial in the ageing process can be determined in relation to levels of hormones. Prevalent age-related diseases are associated with a reduced musculoskeletal system function, notably osteoporosis, osteoarthritis and sarcopenia or with reduced cognitive function, notably several neurodegenerative diseases. Since especially the function of bone tissue, cartilage, skeletal muscle, and the brain are crucial for maintenance of independence into old age, acquiring biomarkers of loss of function of these tissues and organs is particularly desired. Besides the effect of pituitary hormones on tissue maintenance, the effect on physiological parameters, including temperature and ECG measures, could be investigated. Furthermore, tissue-specific levels and effects of these hormones could be determined to identify underlying mechanisms of longevity. To investigate this, tissue-specific knockout mice could be created. Moreover, tissue-specific hormone supplementation could lead to health benefits in contrast to general hormone therapy. It would therefore be worthwhile to investigate the effects of tissue-specific hormone supplementation in animal models.

Studies in laboratory mice already showed that the impact of disrupting GH signalling on longevity is larger than the impact of disrupting IGF-1 signalling or events downstream from IGF-1 receptors, which is in line with our observation that GH secretion was significantly lower in offspring of long-lived families while IGF-1 levels were not-significantly lower. However, nonagenarians with lower IGF-1 bioavailability had improved survival compared to nonagenarians with higher IGF-1 parameters. Future studies should aim to determine whether a reduction in GH or IGF-1 is more important for the longevity phenotype.

Disentangling the underlying mechanisms of the link between reduced GH signalling and longevity is less complicated in animal models than in human. However, how applicable are findings in mice for human? Besides the fact that mice are different organisms than human, laboratory mice live under standardized conditions while human health and lifespan are influenced by many known and unknown factors.

One of the remaining questions is whether the offspring from the Switchbox Study, but also the nonagenarians with improved survival from the Leiden Longevity Study, already had reduced GH/IGF-1 signalling at younger age or whether the offspring and the long-

lived nonagenarians had a steeper decline in their GH/IGF-1 signalling parameters with age compared to controls. When time and money would be no limitation, it is a possibility to follow-up a large number of individuals over time and measure their GH/IGF-1 parameters regularly to look at the change in GH in relation to health and mortality. This is also an option for TSH/TH levels to investigate the change over time and their relationship to health parameters.

The tighter control of GH secretion in offspring of long-lived families is an interesting finding since it suggests that the timing and regulation of a hormone is just as, or even more, important for longevity than total hormone levels. It would be interesting to test in animal models whether this is indeed the case. Moreover, if this stronger regulation leads to preserved resources by secreting the right amount at the right moment and this is a mechanism relevant for longevity, this could also be the case for other systems of the body. This would be an interesting hypothesis to investigate in future studies.

Unravelling the mechanisms of the altered endocrine features of the thyroid and somatotropic axes in human longevity could lead to potential starting points for intervening in the ageing process and will hopefully lead to the improvement of human health at old age.

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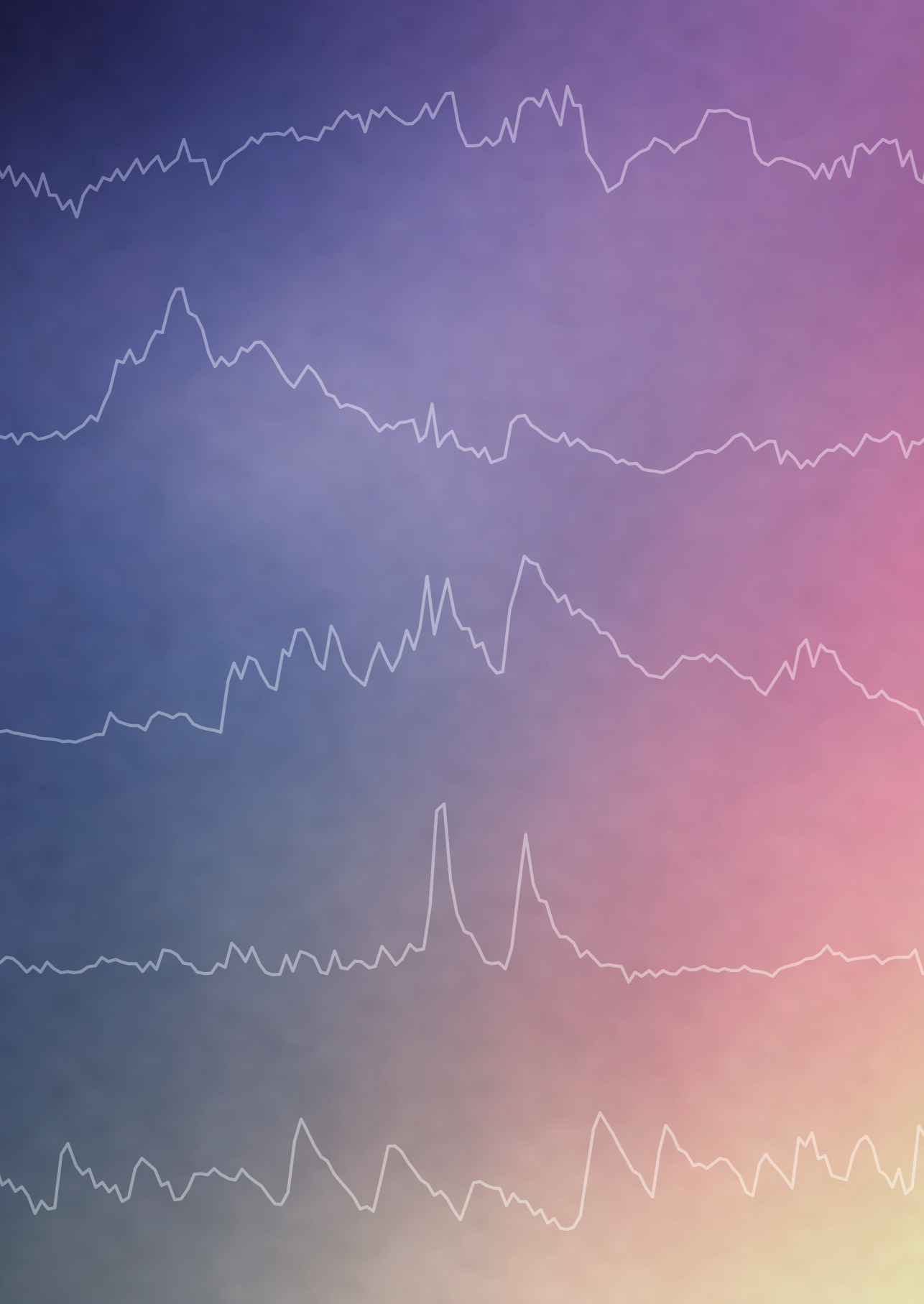


8

English summary

Nederlandse samenvatting

List of publications

List of co-authors

Curriculum Vitae

Dankwoord

ENGLISH SUMMARY

In **Chapter 2**, we investigated whether circulating insulin-like growth factor 1 (IGF-1) axis parameters associate with old age survival and functional status in nonagenarian siblings from the LLS. We found that lower IGF-1/IGF binding protein 3 (IGFBP3) molar ratios were associated with lower hazard ratios, indicating improved survival. Furthermore, nonagenarians in the lowest quartile of IGF-1/IGFBP3 ratios had the highest scores for both Activities of Daily Living (ADL) scales and Instrumental ADL scales, indicating better functional status. In **Chapter 3**, we measured circulating growth hormone (GH) concentrations in blood withdrawn every 10 min and IGF-1 and IGFBP3 every 4 h in healthy offspring of long-lived families and their partners. We found an association between lower total and basal GH secretion and familial longevity. GH secretion was tighter controlled in offspring of long-lived families compared with their partners. No significant differences were observed in circulating levels of IGF-1 and IGFBP3 between offspring and controls, although these tended to be somewhat lower in the offspring. In **Chapter 4**, we investigated in healthy male middle-aged participants whether familial longevity is associated with altered endocrine features in the hypothalamic-pituitary-gonadal axis using luteinizing hormone (LH) and testosterone concentrations measured in blood withdrawn every 10 during 24 h. We found no major differences in the LH and testosterone secretion between male offspring of long-lived families and the control group. In **Chapter 5**, we investigated the interrelationships between hormones from the different hypothalamic-pituitary-target gland axes and confirmed that within interlinked hormonal axes, adrenocorticotropic hormone (ACTH) and cortisol concentrations are correlated, as well as LH and testosterone concentrations. Between hormonal axes, we observed a negative correlation between cortisol and thyroid stimulating hormone (TSH), and a positive correlation between TSH and GH. The joint pattern synchrony between GH and cortisol, and between GH and LH, are the greatest of all hormone combinations. These results are indications that there is interplay between hormonal axes in healthy older individuals. In **Chapter 6**, the 24-h profiles of bone markers in healthy older subjects were determined. We confirmed that C-terminal cross-linked telopeptide of type 1 collagen (CTX) and osteocalcin levels display a circadian rhythm. N-terminal propeptide of type 1 procollagen (P1NP) and sclerostin levels, although not exhibiting a circadian rhythm, varied as well during the day. For Dickkopf-related protein 1 (DKK1), no reliable conclusion on the absence or presence of a 24-h rhythm could be drawn because of the large intraindividual variation. Although levels differed between men and women, timing of the 24-h rhythm of all bone markers did not differ between men and women.

NEDERLANDSE SAMENVATTING

Veroudering

Veroudering gaat gepaard met een achteruitgang in functioneren en een toenemend risico op ziekte en overlijden [1]. Hoe komt het dat we verouderen? Eén van de theorieën is dat dit het gevolg is van de ophoping van moleculaire schade met de leeftijd, wat zorgt voor verstoringen in cellen en weefsels. Dit leidt uiteindelijk tot functionele achteruitgang, ziekte en overlijden [2]. Deze moleculaire schade kan op verschillende manieren ontstaan, zoals door blootstelling aan stoffen van buiten ons lichaam zoals carcinogenen uit ons voedsel, of blootstelling aan stoffen die ontstaan in ons lichaam, zoals toxische bijproducten van onze cellulaire stofwisseling. Het herstellen van schade kost veel energie. Er wordt gedacht dat ons vermogen om schade te herstellen beperkt is omdat een groot deel van onze energie nodig is voor vitale processen zoals groei, ontwikkeling en voortplanting van het organisme. Veroudering is dus mogelijk het gevolg van een disbalans tussen schadeophoping en beperkingen in herstelmechanismen. Veel verschillende interne en externe factoren beïnvloeden deze balans. Hormonen vanuit de hersenen spelen vermoedelijk een belangrijke rol hierin [3].

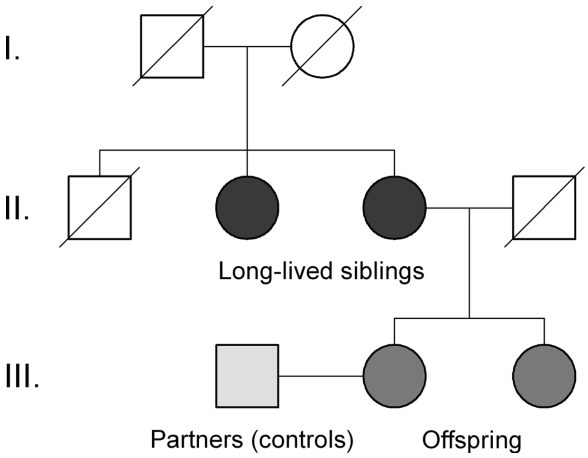
Onderzoek met diermodellen

Eén van die hormonen vanuit de hersenen is groeihormoon. Groeihormoon zorgt via het reguleren van eiwitten van een bepaalde signaalroute (groeihormoon/insulin-like growth factor 1 (IGF-1)) voor groei en ontwikkeling in zoogdieren. Ook in ongewervelde diersoorten komt een soortgelijke signaalroute voor. Onderzoek met verschillende soorten proefdieren laat zien dat er een associatie bestaat tussen deze signaalroute en levensduur [4-11]. De levensduur van de rondworm kan bijvoorbeeld verlengd worden van 2-3 weken naar maximaal 6 maanden als er een mutatie wordt gemaakt in één van de genen die onderdeel zijn van deze signaalroute [12]. Ook in de fruitvlieg zijn mutaties in deze signaalroute, zoals een mutatie in de insulinereceptor, geassocieerd met een verlengde levensduur [6]. Daarnaast hebben meerdere onderzoeken aangetoond dat de levensduur van muizen in het laboratorium verlengd kan worden door verschillende mutaties in de groeihormoon/IGF-1 signaalroute [8].

Leiden Langleven Studie

Er is minder bekend over de rol van de groeihormoon/IGF-1 signaalroute in het reguleren van veroudering en levensduur in mensen. Gezonde veroudering en langlevendheid is in mensen veel lastiger te onderzoeken dan in diermodellen. Naast dat mensen langer leven dan diermodellen, is het moeilijk om een goede controlegroep te vinden op hoge leeftijd.

Daarom worden ouderen vaak met jongeren vergeleken, maar dit kan leiden tot verkeerde interpretaties van de resultaten. Om een aantal van deze tekortkomingen te omzeilen is in 2002 de Leiden Langleven Studie (LLS) opgezet [13]. In deze studie zijn 421 families geïncludeerd op basis van de aanwezigheid van tenminste twee langlevende broers en/of zussen. Mannen moesten minstens 89 jaar oud zijn en vrouwen 91 jaar of ouder en ze moesten tenminste één broer of zus hebben die ook voldeed aan deze leeftijdscriteria. Zie Figuur 1 voor een schematische weergave van de onderzoeksopzet. De nakomelingen van deze negentigjarigen werden eveneens geïncludeerd in dit onderzoek, omdat zij naar verwachting mogelijk ook een hoge leeftijd zullen bereiken. De partners van de nakomelingen werden geïncludeerd als controlegroep. De nakomelingen bleken een lagere prevalentie van hartinfarcten, diabetes mellitus, hoge bloeddruk en metabool syndroom te hebben dan hun partners [14]. Daarnaast werd onder andere gevonden dat het suiker- en vetmetabolisme verschillend was tussen nakomelingen van langlevende families en hun partners [15].



Figuur 1. Onderzoeksopzet van de Leiden Langleven Studie.

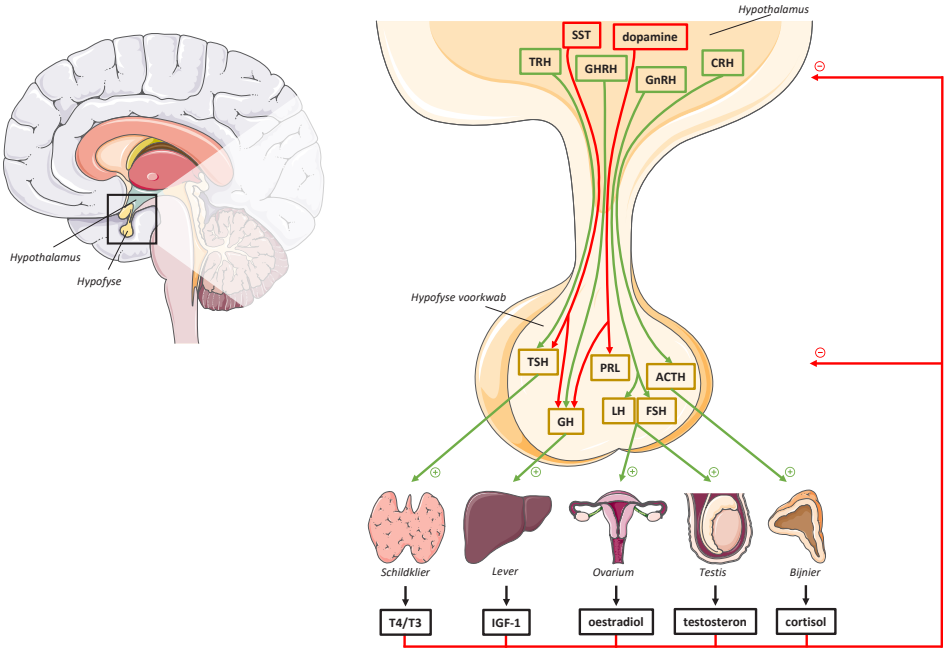
Langlevende families met tenminste twee broers en/of zussen die aan de leeftijdscriteria (mannen ≥ 89 jaar en vrouwen ≥ 91 jaar) voldoen werden geïncludeerd in de LLS samen met hun nakomelingen (offspring) en de partners van deze nakomelingen als controlegroep. Aangepast van *PE Slagboom et al. Phil Trans R Soc Lond B Biol Sci. 2011 Jan 12;366(1561):35-42*.

Hormonen vanuit de hersenen

De resultaten van de onderzoeken in diermodellen en in de LLS hebben geleid tot de hypothese dat hormonen vanuit de hersenen een rol spelen bij (gezonde) veroudering en langlevendheid in mensen. Hormonen vanuit de hersenen zijn onderdeel van de hypothalamus-hypofyse-'eindorgaan' assen. De belangrijkste hypothalamus-hypofyse-'eindorgaan' assen zijn de somatotrope, schildklier, bijnier, gonadotrope en prolactine assen (Figuur 2). Het gemeenschappelijke doel van deze hormonale assen is het behoud van homeostase in het lichaam door het waarnemen en reageren op veranderingen in de omgeving. De associaties tussen hormonen van de schildklieras en bijnieras met familiaire langlevendheid zijn al eerder onderzocht; de TSH-secretie was hoger in de nakomelingen van langlevende families vergeleken met hun partners terwijl niveaus in schildklierhormonen niet verschillend waren [16]. Daarnaast werden er geen verschillen gevonden in de secretie van ACTH (adrenocorticotroop hormoon) en cortisol in rust [17].

Doelen van dit onderzoek

Tijdens mijn promotieonderzoek heb ik de associaties tussen hormonen van de hypothalamus-hypofyse-somatotrope as (doel 1) en de hypothalamus-hypofyse-gonadotrope as (doel 2) met familiaire langlevendheid onderzocht. Daarnaast heb ik onderzoek gedaan naar de onderlinge relaties tussen hormonen van verschillende hypothalamus-hypofyse-eindorgaan assen (doel 3). Hormonen hebben mogelijk een invloed op levensduur door het herstel en behoud van weefsels te reguleren. Daarom heb ik het 24-uurs profiel van biomarkers die iets zeggen over de mate van behoud en herstel van botweefsel onderzocht, zodat deze biomarkers in de toekomst kunnen worden geassocieerd met verschillende hormonen (doel 4). Onderzoek naar de onderliggende mechanismen van de associatie tussen hypofysehormonen en familiaire langlevendheid leidt hopelijk naar manieren om in te grijpen in het verouderingsproces en uiteindelijk tot het verbeteren van de gezondheid op hoge leeftijd.



Figuur 2. Hypothalamus-hypofyse-'eindorgaan' assen.

Schematische weergave van de (regulatie van de) humane hypothalamus-hypofyse-'eindorgaan' assen: hypothalamus-hypofyse-schildklieras, hypothalamus-hypofyse-somatotrope as, hypothalamus-hypofyse-prolactine as, hypothalamus-hypofyse-gonadotrope as en de hypothalamus-hypofyse-bijnieras. De groene pijlen geven de stimulatie van een hormoon weer en de rode pijlen de remming van een hormoon. Plaatjes van organen/weefsels zijn van Servier Medical Art, https://smart.servier.com/. Afkortingen:

ACTH: adrenocorticotroop hormoon
CRH: corticotropine-vrijmakend hormoon
FSH: follikelstimulerend hormoon
GH: groeihormoon
GHRH: GH-vrijmakend hormoon
GnRH: gonadotropin-vrijmakend hormoon
IGF-1: insulin-like growth factor 1
LH: luteïniserend hormoon
PRL: prolactine
SST: somatostatine
T3: tri-joodthyronine
T4: thyroxine
TRH: thyrotropine-vrijmakend hormoon
TSH: schildklier-stimulerend hormoon

1. Hypothalamus-hypofyse-somatotrope as en familiaire langlevendheid

De groeihormoonsecretie wordt gestimuleerd door GH-vrijmakend hormoon (GHRH) vanuit de hypothalamus. Groeihormoon stimuleert de productie van IGF-1 in met name de lever, welke vervolgens zorgt voor negatieve terugkoppeling op de groeihormoonsecretie (Figuur 2). In **hoofdstuk 2** van dit proefschrift hebben we de associatie van IGF-1 parameters met overleving en functionele status op hoge leeftijd onderzocht. Voor dit onderzoek zijn niveaus van IGF-1 en IGF-bindingseiwit 3 (IGFBP3) in het bloed gemeten en vragenlijsten over (instrumentele) algemene dagelijkse

levensverrichtingen afgenomen bij negentigjarigen van de LLS. Vervolgens is de IGF-1/IGFBP3 ratio berekend, wat een maat is voor de biologische beschikbaarheid van IGF-1. De belangrijkste conclusies van dit onderzoek zijn dat negentigjarigen met een lagere IGF-1/IGFBP3 ratio een verhoogde overleving en een betere functionele status hadden dan negentigjarigen met een hogere ratio.

Het is onduidelijk of deze ouderen alleen een lagere beschikbaarheid van IGF-1 hadden op hoge leeftijd of dat ze dit al op jongere leeftijd hadden. We hebben geen gegevens van deze ouderen op jongere leeftijd, maar we hebben wel hun nakomelingen geïncludeerd in de Switchbox studie, welke onderdeel is van de LLS. In deze studie zijn er in totaal 38 gezonde ouderen met een gemiddelde leeftijd van 65 jaar geïncludeerd waaronder 20 nakomelingen van langlevende families en 18 partners. Omdat groeihormoon pulsatiel wordt uitgescheiden en een korte halfwaardetijd heeft, moet het worden gemeten in bloed dat frequent over de dag is afgenomen om betrouwbare informatie te verkrijgen. Daarom is bij deze deelnemers vanaf 9 uur 's ochtends elke 10 minuten gedurende 24 uur bloed afgenomen. De resultaten van dit onderzoek worden besproken in **hoofdstuk 3**. Nakomelingen van langlevende families hadden een lagere groeihormoonsecretie dan hun partners en bovendien was de regulatie van groeihormoon sterker in de nakomelingen. Er zijn geen significante verschillen gevonden in IGF-1 en IGFBP3 niveaus. De sterkere regulatie van groeihormoon zou kunnen betekenen dat groeihormoon alleen wordt uitgescheiden als dit echt nodig is en er zuiniger met de voorraden wordt omgegaan, wat dan ook leidt tot lagere totale secretie. Deze resultaten zijn in overeenstemming met de bevindingen in diermodellen waarin een verlaagde activiteit van de groeihormoon/IGF-1 as tot een verlengde levensduur leidt.

2. Hypothalamus-hypofyse-gonadotrope as en familiaire langlevendheid

LH (luteïniserend hormoon) en FSH (follikelstimulerend hormoon) vanuit de hypofyse stimuleren de productie van de geslachtshormonen testosteron en oestrogeen. Niveaus van de geslachtshormonen dalen met de leeftijd en vooral tijdens de menopauze vindt er een grote daling plaats in vrouwen. Er zijn associaties gevonden tussen geslachtshormonen en langlevendheid in mensen [18, 19]. In **hoofdstuk 4** hebben we in 20 gezonde oudere mannen van de Switchbox studie onderzocht of de LH- en testosteronsecretie geassocieerd is met familiaire langlevendheid. Er zijn echter geen grote verschillen gevonden tussen de groepen. Daarnaast hebben we enkele metingen van oestradiol en prolactine verricht in een 24-uurs pool in alle 38 deelnemers van de Switchbox studie. We hebben in zowel mannen als in vrouwen geen verschillen tussen

nakomelingen van langlevende families en hun partners gevonden in oestradiol en prolactine niveaus.

3. Relaties tussen hormonen van de hypothalamus-hypofyse-'eindorgaan' assen
Als we elke hypothalamus-hypofyse-'eindorgaan' as apart onderzoeken, vinden we dat familiare langlevendheid geassocieerd is met verschillen in de secretie van hormonen van de schildklieras en de somatotrope as. Deze associaties kunnen aparte mechanismen weerspiegelen, maar kunnen ook met elkaar verbonden zijn. Daarom hebben we in **hoofdstuk 5** de onderlinge relaties tussen hormonen van verschillende hypothalamus-hypofyse-'eindorgaan' assen (ACTH, cortisol, groeihormoon en TSH) in gezonde ouderen van de Switchbox studie onderzocht. We hebben onder andere bevestigd dat cortisol- en ACTH-concentraties positief zijn gecorreleerd. Daarnaast vonden we een sterke correlatie tussen TSH- en groeihormoonconcentraties die op hetzelfde moment gemeten zijn. Bovendien waren de hormoonpatronen van groeihormoon en cortisol sterk gesynchroniseerd. Deze resultaten geven aan dat er ook tussen verschillende hormonale assen interactie plaatsvindt. De relaties die we tussen hormonen hebben gevonden waren vergelijkbaar in nakomelingen van langlevende families en hun partners.

4. Biomarkers van weefselbehoud en -herstel
De mechanismen waarop hormonen een rol spelen bij familiaire langlevendheid moeten nog worden ontrafelend. Een hypothese is dat hormonen de balans tussen het investeren van energie in aan de ene kant groei en ontwikkeling en aan de andere kant onderhoud en herstel deels reguleren. De mate van behoud en herstel van weefsels zou kunnen worden bepaald aan de hand van gevalideerde biologische markers (biomarkers) van weefselbehoud en -herstel die te meten zijn in bijvoorbeeld bloed of urine. Om deze te kunnen vinden, hebben we een review over mogelijke biomarkers van behoud en herstel van bot-, kraakbeen-, spier- en hersenweefsel geschreven (niet in dit proefschrift). Voordat deze biomarkers van weefselherstel gebruikt kunnen worden in onderzoek, moeten eerst een aantal praktische zaken worden onderzocht, waaronder het circadiaans ritme van de biomarkers. Omdat er van botweefsels al gevalideerde biomarkers beschikbaar zijn, hebben we in **hoofdstuk 6** van dit proefschrift het 24-uurs profiel van vijf verschillende biomarkers van botweefselbehoud en -herstel onderzocht. We hebben deze biomarkers op zes tijdspunten binnen 24 uur gemeten in 38 gezonde ouderen van de Switchbox studie. Zowel de botresorptiemarker CTX (C-terminal cross-linked telopeptide of type 1 collagen) als de botformatiemarker osteocalcine hebben hogere niveaus in de nacht vergeleken met de dag. Ook de botformatiemarker P1NP (N-terminal propeptide of type 1

procollagen) heeft hogere niveaus in de nacht vergeleken met de dag, maar dit werd alleen in mannen gevonden en niet in vrouwen. Sclerostine en DKK1 (Dickkopf-related protein 1), die de botformatie remmen, varieerden over de 24 uur, maar niet volgens een duidelijk circadiaans ritme. Dit onderzoek laat zien dat het belangrijk is om de meeste botmakers gestandaardiseerd en frequent te meten vanwege de fluctuaties over een etmaal.

Op dit moment is het niet bekend of juist lagere of hogere niveaus van biomarkers van weefselbehoud gunstig zijn. Lagere niveaus zouden er op kunnen duiden dat weefsels zich niet meer kunnen herstellen, maar het kan ook betekenen dat cellen alleen vernieuwd worden als dit echt nodig is, wat zou kunnen leiden tot een langer vermogen om weefsels te herstellen. Hogere niveaus kunnen er op duiden dat weefsels in staat zijn om te herstellen, maar stamcelreserves kunnen hierdoor ook eerder uitgeput raken. Om dit te kunnen bepalen, zouden deze biomarkers gemeten kunnen worden in mensen met verschillende biologische leeftijd.

Toekomstperspectieven

Vervolgstudies zouden erop gericht moeten zijn om de onderliggende mechanismen van de associatie tussen hypofysehormonen en familiaire langlevendheid te ontrafelen. Hopelijk leidt dit tot manieren om in te grijpen in het verouderingsproces en uiteindelijk tot het verbeteren van de gezondheid op hoge leeftijd.

Naast het effect van hormonen op weefselbehoud en -herstel zou het effect op fysiologische parameters, zoals ECG-parameters en lichaamstemperatuur, kunnen worden onderzocht. Als het mogelijk is, zou er ook naar weefselspecifieke niveaus en effecten van hormonen moeten worden gekeken aangezien deze afwijken van de niveaus in het bloed, maar mogelijk een grote invloed op veroudering en langlevendheid hebben.

Familiaire langlevendheid is geassocieerd met verschillen in de TSH- en groeihormoonsecretie, maar het is niet duidelijk welk van deze twee hormonen belangrijker is voor langlevendheid of dat TSH en groeihormoon samenwerken. Omdat de Switchbox studie een cross-sectioneel en observationeel onderzoek is, konden de directe effecten van hormonen op elkaar niet worden onderzocht. Om te onderzoeken wat een bepaald hormoon voor invloed heeft op andere hormonen zouden er interventiestudies kunnen worden uitgevoerd.

Onderzoek in muizen heeft aangetoond dat de impact van verminderde groeihormoonactie groter is dan de impact van verminderde IGF-1 actie op levensduur. Vervolgstudies zouden kunnen onderzoeken welk hormoon belangrijker is voor het langleven fenotype in mensen. Daarnaast is het interessant om te onderzoeken in hoeverre de resultaten die gevonden zijn in muizen overeenkomen met onderzoeksresultaten van humane studies. Muizen zijn niet alleen andere organismen, maar leven ook onder gestandaardiseerde omstandigheden terwijl de gezondheid en levensduur van mensen door een groot aantal (on)bekende factoren beïnvloed wordt.

Een andere vraag is of de nakomelingen van de Switchbox studie, maar ook de langlevende negentigjarigen van de LLS, al lagere groeihormoon/IGF-1 niveaus hadden op jonge leeftijd of dat ze een sterkere daling in hun groeihormoon/IGF-1 niveaus hadden met de leeftijd. Een studiepopulatie zou over de tijd moeten worden gevolgd om de veranderingen in groeihormoon/IGF-1 niveaus in relatie tot gezondheid en veroudering te onderzoeken. Dit is ook interessant voor andere hormonen zoals TSH en schildklierhormonen in relatie tot gezondheidsparameters.

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CURRICULUM VITAE

Evelien (Evie) van der Spoel is op 2 augustus 1992 geboren in Rotterdam. Ze is opgegroeid in Rhoon en heeft in 2010 haar vwo-diploma behaald aan het PENTA college CSG Blaise Pascal in Spijkenisse. Vervolgens is ze Biomedische Wetenschappen gaan studeren aan de Universiteit Leiden. In het derde jaar volgde ze de minor Medische Technologie aan de TU Delft. Haar bachelorstage heeft ze op de afdeling Interne Geneeskunde, sectie Ouderengeneeskunde, van het Leids Universitair Medisch Centrum (LUMC) onder begeleiding van dr. ir. D. van Heemst uitgevoerd. Tijdens deze stage is zij betrokken geweest bij de dataverzameling van de Switchbox studie en heeft ze het verschil in energiemetabolisme tussen nakomelingen van langlevende families en hun partners, en tussen jongeren en ouderen, onderzocht. Vervolgens is ze hier als onderzoeksassistent blijven werken om algemene onderzoekstaken uit te voeren en om mee te werken aan diverse publicaties.

In september 2013 begon zij met de masteropleiding Biomedische Wetenschappen aan de Universiteit Leiden. Ze volgde keuzevakken aan de Leyden Academy of Vitality and Ageing, het Nederlands Kanker Instituut en de Universiteit Leiden. Haar eerste masterstage heeft ze op de afdeling Parasitologie van het LUMC onder begeleiding van dr. B. Guigas en dr. H.H. Smits uitgevoerd, waar ze het metabolisme van regulatoire B-cellen tijdens een Schistosoma infectie heeft onderzocht. Voor haar afstudeerstage keerde zij terug naar de afdeling Interne Geneeskunde, sectie Ouderengeneeskunde. Tijdens deze stage deed ze onderzoek naar de associatie tussen groeihormoonsecretie en familiare langlevendheid onder begeleiding van dr. ir. D. van Heemst en dr. S.W. Jansen. Eind 2015 ontving zij een BW-plus beurs van de Raad van Bestuur van het LUMC om drie jaar promotieonderzoek te doen.

Na het behalen van haar masterdiploma is Evie in januari 2016 begonnen als promovenda bij de sectie Ouderengeneeskunde binnen de afdeling Interne Geneeskunde van het LUMC onder begeleiding van prof. dr. G.J. Blauw, prof. dr. H. Pijl (sectie Endocrinologie) en dr. ir. D. van Heemst. Resultaten van dit onderzoek zijn beschreven in dit proefschrift. In mei 2019 is zij begonnen als postdoctoraal onderzoeker binnen dezelfde sectie als waar ze haar promotieonderzoek heeft uitgevoerd.

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